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Research under current project has been carried out in three interconnected directions aimed to develop new therapeutic approaches against biological weapon threat agents, namely Bacillus anthracis and smallpox virus (using vaccinia virus model).

We have successfully demonstrated new previously unexplored directions to improve anthrax antibiotic therapy by using a combination of antibiotic with apoptosis inhibitors and adenosine receptor agonists.

New candidate virulence factors of B. abthracis have been suggested based on our bioinformatics analyses and experimental results.

Evidence on toll-like receptor participation in both infections caused by B. anthracis and vaccinia virus has been obtained.

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INTRODUCTION

In this report, we are suggesting several promising and novel approaches for prophylaxes and treatment of diseases resulting from BW that could significantly increase the survival rates of individuals infected with biological warfare agents.

The three tasks we report are based on *in vitro* and *in vivo* experiments that have been performed by NCBD. The research has been conducted to determine the best substances, approaches, and optimal qualitative and quantitative parameters of the proposed therapies.

BODY OF THE REPORT

TASK 1. SYNERGISTIC ANTI-TOXIN/ANTIBIOTIC TREATMENT OF ANTHRAX

1.1. Protective Efficacy Evaluation Of Caspase Inhibitors And Immunostimulators Using A Murine Animal Model

1.1.1. Background

The causative agent of anthrax, *B. anthracis*, is a Gram-positive, spore-forming organism that generally infects herbivores (Hanna, 1998). Self-limiting infections in humans most often result from the introduction of spores through lesions in the skin, though the highly lethal form of anthrax is caused by inhalation of spores. In inhalational anthrax, spores are engulfed by alveolar macrophages, which carry the spores to the mediastinal lymph nodes. The spores germinate inside the migrating macrophages, producing an antiphagocytic capsule and two toxins: lethal toxin (LeTx) and edema toxin (EdTx). Lysis of the macrophages allows release and proliferation of the bacteria in the lymphatic system, reaching concentrations of 10^4-10^5 bacteria per ml of lymphatic tissue. The bacteria then escape into the bloodstream and continue to proliferate. Bacteremia rises steadily until the last few hours before death, reaching 10^8-10^9 bacteria per ml of blood (Smith *et al.*, 1955; Hanna et al., 1993). Death is attributed to severe respiratory distress and multi-system organ failure caused by sepsis and septic shock. These conditions have been poorly studied and are generally assumed to result from the overproduction of pro-inflammatory cytokines and other mediators.

The virulence of anthrax is determined primarily by its toxins and in particular LeTx. Therefore, the toxins have been the subject of the majority of research on anthrax treatment and prevention. Current theory centers on LeTx function in late infection, when the toxin released into the bloodstream is implicated in the development of anthrax sepsis, septic shock, and death. The "extracellular" model of LeTx action suggests that LeTx attacks sensitive cells by binding to a putative cell surface receptor and ultimately translocating the lethal factor (LF) toxin subunit into the cell cytosol. However, it has recently been observed that the toxin genes and their *trans* activator, atxA, are expressed within the macrophage immediately after spore germination (Guidi-Rontani *et al.,* 1999). Recent studies (Pellizzari *et al.,*

1999; Erwin *et al.,* 2001) have shown that LeTx does not cause the overproduction of pro-inflammatory cytokines and reactive oxygen species reported previously (Hanna *et al.,* 1994). Also, we have demonstrated that macrophages treated with LeTx do not induce the release of IL-1β, TNF-α and are unable to respond to the stimulation with innate antigens, such as bacterial cell wall (CW) (Popov *et al.*, 2002b). The pathogenic mechanisms of anthrax toxins warrant further investigation.

Our data (Popov *et al.*, 2002a) suggest a novel mechanism of LeTx-induced cell death that may contribute significantly to the pathology of anthrax. A previously unrecognized function of LeTx is the suppression of the anti-microbial function of host macrophages. In addition to its role as exotoxin, LeTx can be considered as an early intracellular virulence factor that is secreted by vegetating bacilli inside macrophages within a few hours after the spores enter the host. We have concluded that in the early stages of disease, the LeTx becomes active inside the infected macrophage, promoting both intracellular bacterial replication and macrophage apoptosis (Popov *et al.*, 2002 a, b). Recently Park *et al.* (Park *et al.*, 2002) confirmed our findings and discovered p38 MAPK signaling cascade as a target of LeTx.

Our data indicate that the apoptotic signaling through the TNF-like Fas receptor and its ligand (FasL) is involved in LeTx-induced macrophage death. This finding may have several important implications and opens new avenues for therapeutic interventions. Apoptotic macrophages are capable of the fast release of toxic soluble mediators, such as soluble FasL (Kiener *et al.*, 1997), and killing nearby cells (Brown and Savill, 1999). This signaling in response to LeTx may provide a mechanism for amplifying the initial effect of toxin. Soluble FasL is toxic at low doses (Tanaka *et al.*, 1997) and can cause different pathological conditions relevant to anthrax, such as acute respiratory distress syndrome (Matute-Bello *et al.*, 1999; Matute-Bello *et al.*, 2001; Serrao *et al.*, 2001), systemic tissue injury (Tanaka *et al.*, 1995), and neurotoxicity (Fish *et al.*, 1968; Chiarugi *et al.*, 2001).

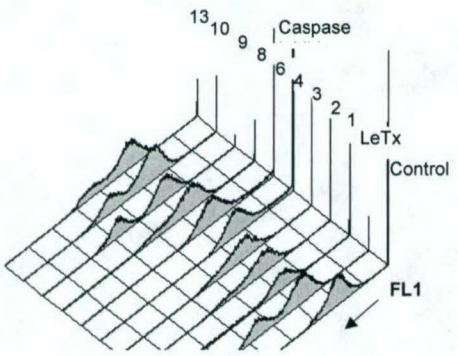


Fig. 1.1. Inhibition of caspases decreases LeTx pathogenicity. Apoptosis is carried out via the action of a number of initiator and effector caspases (Rathmell and Thompson, 1999). Inhibition of caspases using specific inhibitors of caspase-1, -2, -3, -4, -6, and -8 leads to different degrees of cellular protection against LeTx, with inhibition of caspase-4, -6, and -8 being the most effective.

Apoptosis in anthrax is relevant to both the early intracellular stage of anthrax, when macrophages serve as places of spore germination, bacilli multiplication and toxin expression, as well as to the late septicemic stage characterized by high bacteremia accompanied by secretion of toxins from bacilli (Popov *et al.,* 2004). We therefore hypothesized that anti-apoptotic treatment would be beneficial for anthrax prophylaxis and treatment, and tested several model compounds, such as caspase inhibitors and adenosine receptor agonists, in murine model. Preliminary rationale for these experiments came from our in vitro studies, which demonstrated that caspase inhibitors specific for a number of caspases protected macrophage-like cells in culture from LeTx-induced apoptosis (Fig.1.1).

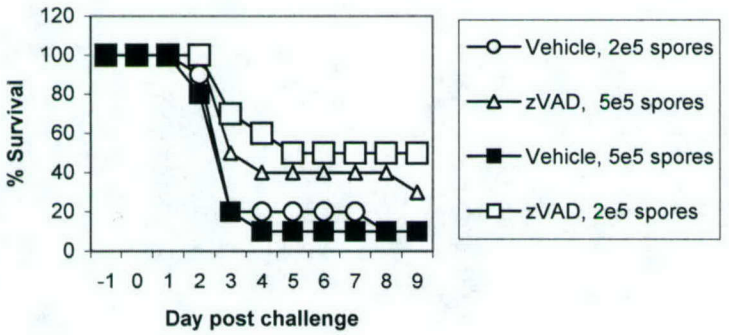


Fig.1.2. Survival of A/J mice challenged i.p. with *B. anthracis* (Sterne) on day 0 and treated i.p.with z-VAD-fmk at days –1, 0, 1 and 4 twice daily (20 mg/kg/day).

We also found that liver cell apoptosis is prominent in late anthrax (Popov *et al.,* 2004). It was accompanied by IL-1β systemic release, indicating that inhibition of caspase-1 (ICE, interleukin-1-converting enzyme) may represent an effective way of preventing liver failure during the infectious process. For this purpose we chose the caspase-1/4 inhibitor YVAD. Previously, we demonstrated positive therapeutic effect of a general caspase inhibitor z-VAD (Fig. 1.2). We also reported the successful application of an adjunct therapy against anthrax infection targeting both bacterial multiplication and host response to infection by using a combination of antibiotic and caspase inhibitors. Up to now, there has been no other report on any anti-LT treatment during the anthrax infectious process.

Data in the literature demonstrated that the A3 receptor (A3AR) agonist N6-(3-iodobenzyl) adenosine-5'-N-methyluronamide (IB-MECA) protected cardiomyocytes in culture from apoptosis (Goldenberg *et al.,* 2003). Previously, Fishman et al. (2000) showed that adenosine and its synthetic analogs (IB-MECA and Cl-IB-MECA) were able to induce G-CSF production from PBMCs. This finding

supported our hypothesis, because we previously found that hematopoietic factors protected cells from LeTx (S. Popov, K. Alibek, unpublished). The oral availability of the A3AR agonists, and their induced systemic anticancer and myeloprotective effects, render them potentially useful in different modes of treatment. We were especially interested in a combination treatment with antibiotic to enhance the therapeutic index of anti-apoptotic substances.

1.1.2. Results

In this report we tested caspase inhibitors as well as A3AR agonists separately and in combination with each other in mice challenged with *B. anthracis* (Sterne). Inhibition of secreted pathogenic factors is not expected to directly interfere with bacterial multiplication and therefore may not be fully protective. In order to target both the bacteria and the proteolytic factors we used a combination therapy where antibiotic administration was complemented by the administration of a protease inhibitor. We were also interested in the efficacy of delayed treatment initiated after certain periods of time following spore challenge. It is a practically relevant scenario because patients generally seek medical help after the onset of symptoms, and in other patients treatment begins after a certain period of time required to confirm the exposure. In addition, a delayed ciprofloxacin therapy in murine model is only partially protective when currently recommended human antibiotic doses (adjusted for body weight) are used in mice (Popov *et al.*, 2004). It seemed interesting to study if a combination approach could lead to a synergistic enhancement in survival.

Animals received ac-YVAD-cmk, IB-MECA or Cl-IB-MECA on days +1 to +4 once daily, s.c. Mice were challenged with anthrax spores ($1x10^7$ spores, i.p.) on day 0. Survival of animals was monitored for 15 days. Ciprofloxacin treatment (50 mg/kg, once daily, i.p.) was initiated simultaneously with the administration of inhibitors, and continued for 14 days. Two doses of YVAD (2.5 mg/kg and 12.5 mg/kg) and 3 doses of IB-MECA or Cl-IB-MECA (0.05, 0.15 and 0.3 mg/kg) were chosen for this study. Figs. 1.3 shows that all animals challenged with *B.anthracis* died by day 6, and YVAD administration did not improve survival, although the higher dose delayed death by 3 days. This result is consistent with our expectations, because only higher doses of z-VAD were partially protective in previous experiments (Fig.1.1).

A combination of YVAD with ciprofloxacin demonstrates a synergistic increase in animal survival up to 70% compared to 30% for antibiotic alone. This data support our hypothesis on liver-protective action of caspase-1 inhibitor, and

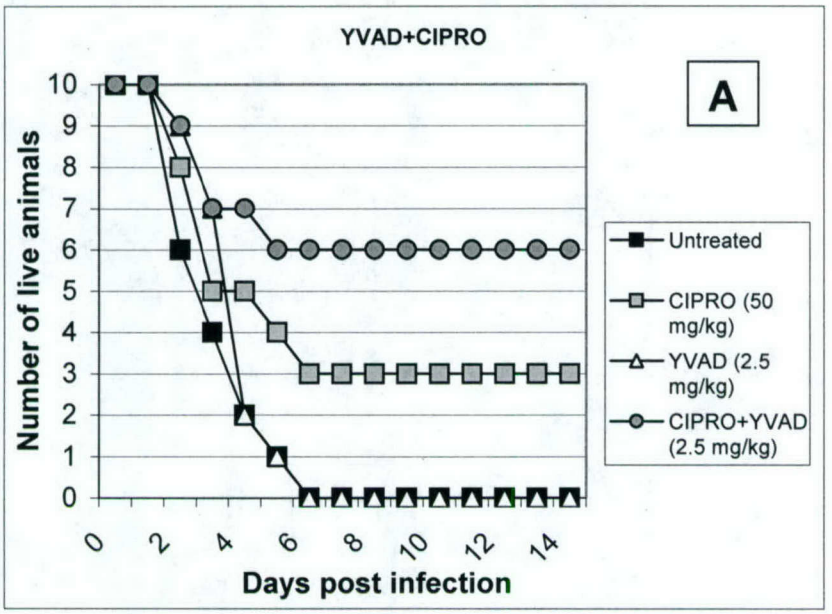
argue in favor of further development of anti-apoptotic approaches to anthrax treatment.

Treatment with Cl-IB-MECA alone (Fig.1.4 A) shows a complex dependence of survival on the inhibitor dose. A 40% protection takes place at 0.15 mg/kg while higher and lower doses are ineffective. We suggest that the ability of this inhibitor to cause massive histamine release from mast cells can, at least in part, can be taken into account in order to explain the above results (Smith *et al.,* 2002). Combinations of different Cl-IB-MECA doses with ciprofloxacin are more effective than the antibiotic alone, however the difference is statistically reliable ($p<0.05$) only for the 0.3 mg/kg dose of Cl-IB-MECA.

We further investigated triple combinations of YVAD, Cl-IB-MECA and ciprofloxacin (Fig. 1.5). While combinations of YVAD, Cl-IB-MECA, tested at two doses, are marginally protective, a very strong synergistic effect takes place with ciprofloxacin (90% protection in Fig. 1.5 A). Higher doses were less effective (Fig. 1.5 B).

1.1.3. Conclusions and suggestions

Overall, these results are remarkable because they emphasize the importance of the host response modulation in anthrax treatment. The inhibitors we tested have no direct antimicrobial function. Instead, both the caspase inhibitor and the A3R agonist are capable of modulating levels of Il-1β, TNF-α, IL-12, IL-10 and G-CSF, along with the level of histamine and the apoptotic mediators, such as FasL. In our opinion, further development in this direction holds a big promise.



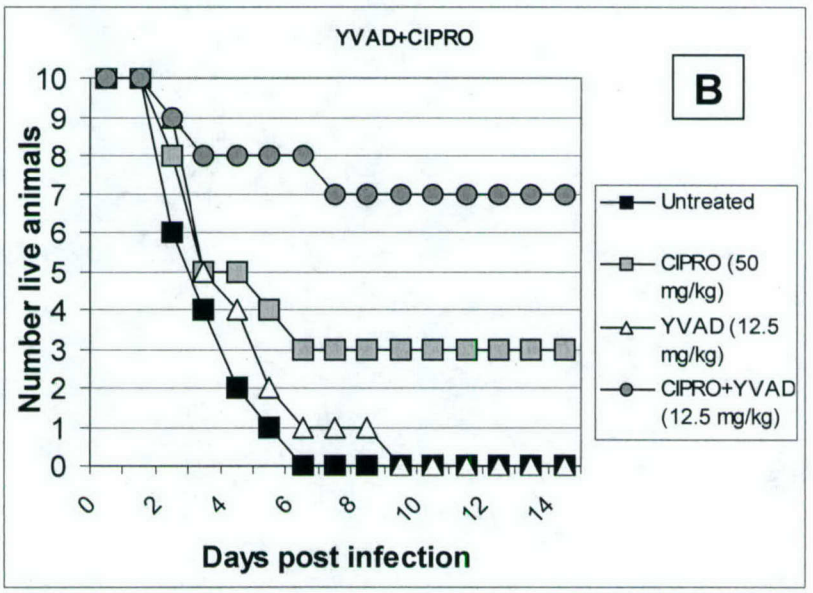


Fig.1.3. Survival of DBA/2 mice after treatment with caspase-1 inhibitor YVAD (A, 2.5 mg/kg; B, 12.5 mg/kg) in combination with ciprofloxacin.

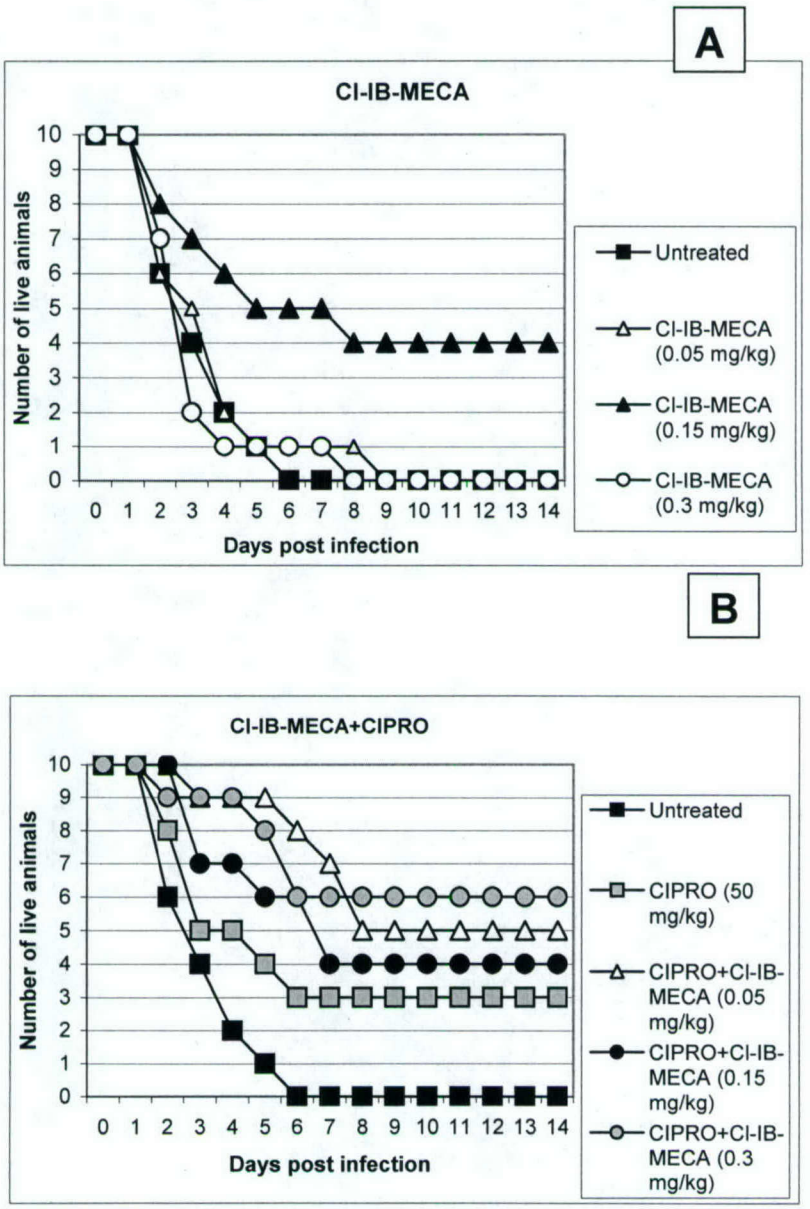


Fig.1.4. Survival of DBA/2 mice after treatment with A3R agonist Cl-IB-MECA alone (A) and in combination with ciprofloxacin (B).

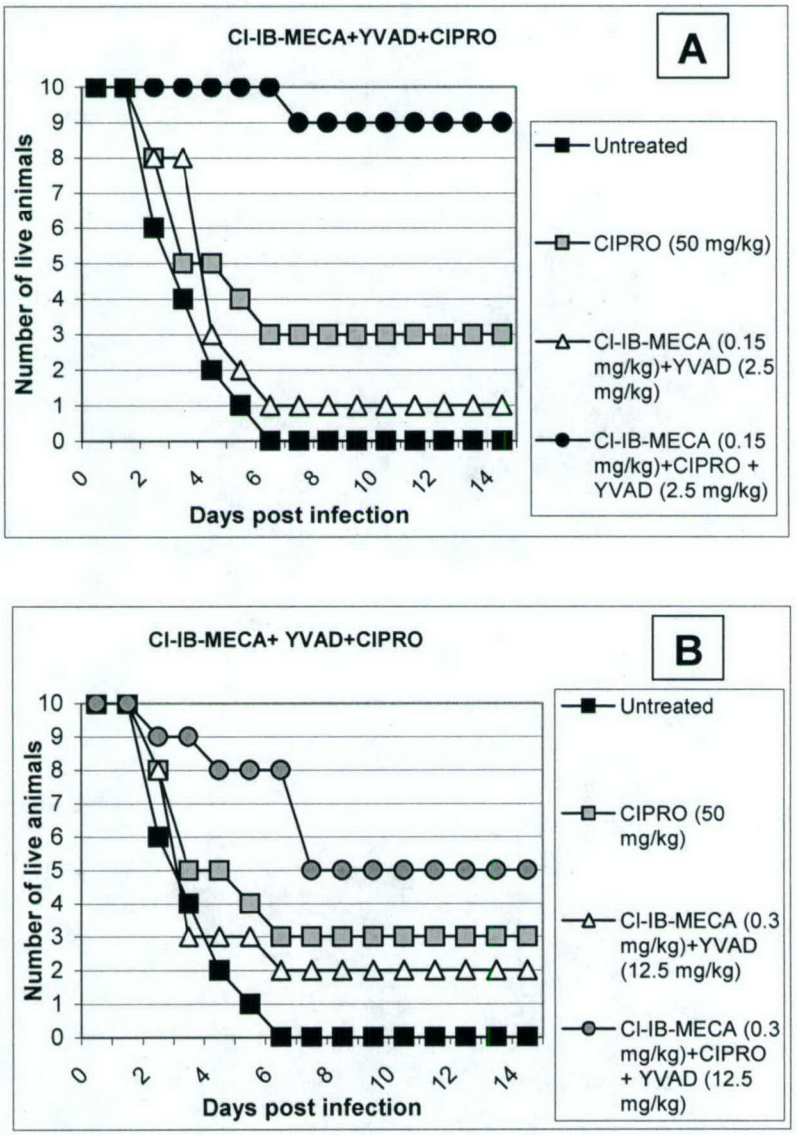


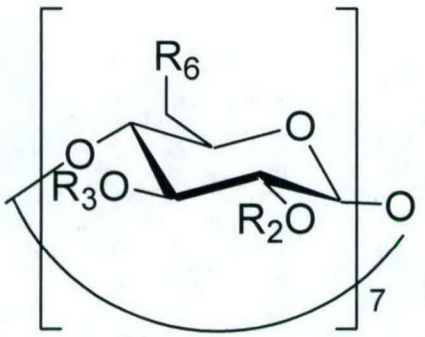
Fig.1.5. Survival of DBA/2 mice after treatment with a triple combination of caspase-1 inhibitor YVAD (2.5 mg/kg, A; 12.5 mg/kg, B), A3R agonist Cl-IB-MECA (0.15 mg/kg, A; 0.3 mg/kg, B) and ciprofloxacin 50 mg/kg).

1.2. Synthesis And Testing Of Alkylamino Derivatives as Inhibitors of Anthrax Toxins

1.2.1. Background

Previously, in a separate study supported by the NIH, we demonstrated that small molecular weight compounds designed to block the trans-membrane pore formed by *B. anthracis* protective antigen could inhibit anthrax lethal toxin (LeTx) action *in vitro*. We showed that per-substituted alkylamino derivatives of β-cyclodextrin displayed inhibitory activity, and they were protective against LeTx action at low micromolar concentrations. Our goal in this study was to test their effectiveness *in vivo*.

One of the alkylamino derivatives: per-6'-propylamino-β-cyclodextrin (PrAmBC) (Fig.1.6) was synthesized by Pinnacle Pharmaceuticals, Inc. (VA).



R_2	R_3	R_6
H	H	$SCH_2CH_2CH_2NH_2$

Fig. 1.6. Structure of per-6'-propylamino-β-cyclodextrin (PrAmBC).

1.2.2. Results

The ability of PrAmBC to inhibit anthrax lethal toxin action was demonstrated on murine macrophage-like RAW 264.7 cells (Fig.1.7).

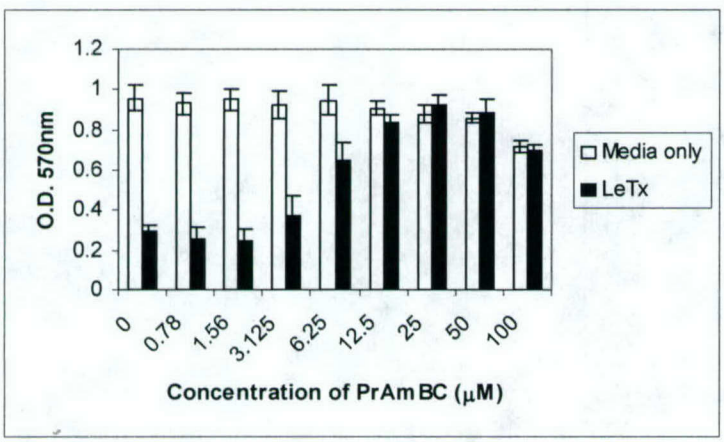


Fig. 1.7. Protection of RAW 264.7 cells from LeTx-induced cell death by PrAmBC. RAW 264.7 cells were incubated with different concentrations of the PrAmBC with or without LeTx. Cell viability was determined in triplicates by MTS colorimetric assay. Error bars represent standard deviations.

In vivo toxicity tests of PrAmBC were performed in murine model. DBA/2 mice (5 per group) were injected intraperitoneally (i.p.) with different doses of PrAmBC (2.5; 10 and 25 mg/kg) once a day for five days. The mice were monitored daily to determine survival. The toxicity tests demonstrated that the compound was toxic at 25 mg/kg (1 of 5 mouse died, and the rest of animals in this group looked very sick) but the mice survived 10 mg/kg dosage which was chosen for the efficacy studies.

In vivo efficacy studies of PrAmBC were performed using DBA/2 mice (10 per group) challenged (i.p.) with 5 x 10^6 spores of *B. anthracis* Sterne strain per mouse. Control mice received PBS (i.p.) daily. In the groups receiving the compound alone or with antibiotic, the mice were injected with 10 mg/kg of PrAmBC (i.p.) once a day. Ciprofloxacin was administered subcutaneously (s.c.) daily in the groups receiving antibiotic alone or with PrAmBC. The mice were monitored daily to determine survival. The results are presented on Fig. 1.8.

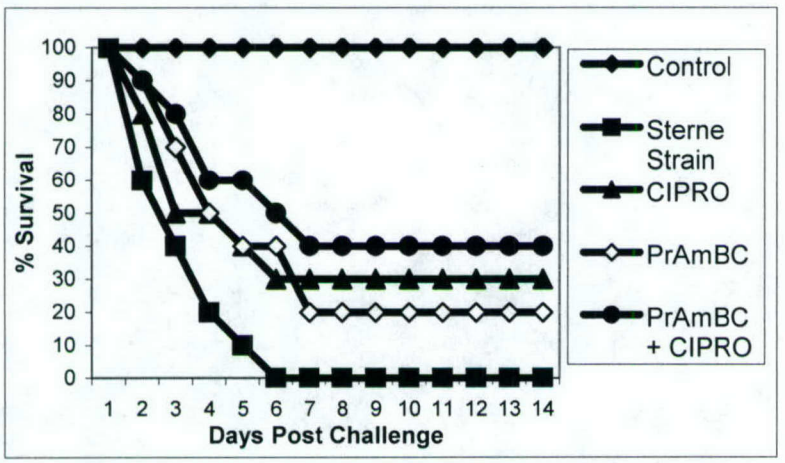


Fig. 1.8. Protection of DBA/2 mice against anthrax infection using ciprofloxacin in combination with PrAmBC.

All untreated animals died. Treatment with PrAmBC in combination with ciprofloxacin resulted in 40% survival of mice compared with 20% for PrAmBC alone and 30% for ciprofloxacin alone treatment. These differences in survival are statistically unreliable ($p>0.1$)

1.2.3. Conclusions

The data demonstrate that per-substituted alkylamino derivatives of β-cyclodextrin can protect macrophages in vitro, however their effect on anthrax infection is marginal. Further improvement of the compounds and modifications in challenge and treatment schedules are necessary.

TASK 2. ANTHRAX PROTEASE INHIBITORS FOR THERAPY OF LATE-STAGE INHALATIONAL ANTHRAX

2.1. Background

Inhalation anthrax is characterized by systemic spread of the challenge agent, *Bacillus anthracis*, which is capable of causing severe damage to all host tissues and organs. Multiple hemorrhagic lesions in the mediastinum, mediastinal lymph nodes, bronchi, lungs, heart, spleen, liver, intestines, kidneys, adrenal glands, and/or central nervous system are typically found upon postmortem examination of patients who succumbed to inhalation anthrax. The most dramatic and potentially life-threatening changes were observed in the vascular system with a diffuse vasculitis extending from moderate sized arteries and veins down to the capillary level. The vasculitis was often associated with vessel destruction, especially of the smallest vessels, and was typically accompanied by massive necrosis in some tissues (Abramova *et al.*, 1993; Grinberg *et al.*, 2001, Vasconcelos *et al.*, 2003). It is widely believed that anthrax lethal toxin (LT) secreted by proliferating bacteria is a major cause of death in man and in several other susceptible animal species (Inglesby *et al.*, 2002). However, the pathology of intoxication in experimental animals is drastically different from that found during the natural infectious process . Recent extensive analyses in mice and rats challenged with a highly purified lethal toxin (Moayeri *et al.*, 2003, Chui *et al.*, 2004) confirmed earlier observations (Klein *et al.*, 1966) that toxin activity caused no gross pathology and almost solely manifested in hypoxic liver failure. In addition to lethal toxin, the hemorrhagic and other tissue-damaging factors elaborated by *B. anthracis* could play important virulence-enhancing roles but these factors have not yet been characterized. In fact, early publications using culture filtrates of *B. anthracis* assumed that the observed effects of secreted substances were caused by LT (Eckert and Bonventre, 1963; Ezepchuk *et al.,* 1969). It is also possible that these factors could themselves exhibit a direct lethal effect.

The capacity of bacteria to cause destruction of tissues, as well as other pathological consequences such as degradation of immunoglobulins, cytokines and complement, release of inflammatory mediators, or activation of host proteolytic enzymes, is attributed to a wide variety of secreted proteases (Supuran *et al.*, 2002), however in the case of *B. anthracis* the proteases of this microorganism, other than lethal factor, have attracted little attention in the scientific literature. The current study aimed to carry out an initial characterization of certain *B. anthracis* proteolytic enzymes and to evaluate their pathogenic role.

Complete genome sequences of two virulent anthrax strains are now available (Read *et al.*, 2002; Read *et al.*, 2003), in addition to the sequences of two avirulent species from the same family, namely *B. cereus* (Ivanova *et al.*, 2003) and *B. subtilis* (Kunst *et al.*, 1997). This information, along with the known sequence motifs characteristic of hundreds of families of proteolytic enzymes

(MEROPS database; Barrett, 2004), allows a whole-genome level comparative analysis of all protease genes that are present in genomes of the three species. The evidence presented here shows that the most potent proteases secreted by *B. anthracis* in culture are represented by metalloprotease (MP) enzymes which act primarily as collagenases. In addition, several closely related thermolysin-like MPs of the M4 family are candidate enzyme capable of causing a hemorrhagic effect similar to thermolysin (EC 3.4.24.27) from *Bacillus thermoproteolyticus*. Moreover, these MPs are more abundant in both *B. anthracis* and *B. cereus*, compared to the avirulent *B. subtilis*.

B. anthracis strain delta Ames is devoid of both toxigenic plasmids and produces neither lethal nor edema toxins. Nevertheless, we demonstrate that the proteins secreted by this strain are directly lethal to mice upon intra-tracheal administration at doses as low as 500 mg/kg. The death of these animals can take place as quickly as a few hours after administration of the proteins.

In comparison, lethal toxin in similar condition is non-lethal. In order to evaluate the role of secreted MPs as virulence factors in the anthrax infectious process we used well-known protease inhibitors (phosphoramidon and 1,10-phenanthroline), as well as polyclonal rabbit antisera raised against peptides representing common motifs of several *B. anthracis* MPs, for treatment of mice challenged with *B. anthracis* (Sterne) spores. The inhibitors alone were ineffective in protecting mice, while a post exposure administration of immune serum to challenged mice demonstrated substantial protective effect (30 to 60%) even without antibiotic, depending on the dose and specificity of a particular antiserum. Both a strong synergistic enhancement of survival rates (up to 90% protection) and a faster recovery were observed when, in addition to either the inhibitor or the serum therapy, mice received antibiotic treatment, which alone protected only 20% mice in the conditions of our experiments. We conclude that secreted virulence factors other that lethal toxin play an important role in anthrax and therefore have to be targeted by a combination post-exposure therapy.

2.2. Results

Genomic analysis of B. anthracis secreted proteins as potential virulence factors. In order to evaluate a pathogenic potential attributed to the *B. anthracis* proteins other than known lethal and edema toxins we used a nontoxigenic and nonencapsulated strain of *B. anthracis* (delta Ames), which is a parental Ames strain cured of both plasmids, pXO1 and pXO2. The substances secreted by vegetative *B. anthracis* cells seem to be the most promising candidates, as is the case for many bacterial toxins (Supuran *et al.*, 2002). Analysis of the chromosome sequence of the *B. anthracis* Ames strain revealed a variety of potential virulence-enhancing factors, including collagenases, phospholipases, haemolysins, proteases and other enterotoxins identified based on their sequence homology with pathogenic factors in other bacterial species (Read *et al.*, 2003). The *B. cereus* group, which includes *B. anthracis, B. thuringiensis and B. cereus,* has an expanded

number of predicted secreted proteins relative to nonpathogenic *B. subtilis* (Read *et al.*, 2003). These *B. cereus* group-specific genes represent the ancestral adaptations to a pathogenic lifestyle by the common ancestor, which was quite similar to *B. cereus*. Our attention was attracted to the group of proteases that are encoded on the *B. anthracis* chromosome, shared in common with *B. cereus* but absent or relatively rare in the genomes of nonpathogenic bacteria. A large number of these proteases fall into clan MA [classified according to the MEROPS system; Barrett AJ, 2004], which among others includes thermolysin-like enzymes of the M4 family. Metalloproteases (MPs) from several bacterial species belonging to this family are capable of causing massive internal hemorrhages and other life-threatening pathologies (Supuran *et al.*, 2002; Sakata *et al.*,1996; Shin *et al.*, 1996; Miyoshi *et al.*, 1998; Okamoto *et al.*, 1997).

Whole genome analyses also indicated collagenolytic proteases of the M9B family as potentially having pathogenic functions. Eleven protease families are present in *B. anthracis* and *B. cereus* but absent in *B. subtilis*. Six of the eleven subfamilies encode MPs. Three of the latter, namely M6, M9B, and M20C subfamilies, are encoded on the bacterial chromosomes. Members of the M6 peptidase family are usually annotated as "immune inhibitors" because in *B. thuringiensis* they can inhibit the insect antibacterial response (Lovgren *et al.*, 1990). The M20C peptidase subfamily represents exopeptidases (Biagini *et al.*, 2001) that are the unlikely cause of tissue destruction or internal bleeding. Based on the above analysis, this study focused on the M4 family thermolysin/elastase-like neutral proteases and the M9 family collagenases as the candidate virulence-enhancing factors of *B. anthracis* Aimes strain.

Hemorrhagic, caseinolytic and gelatinolytic activities of anthrax proteases. The proteins secreted by three *Bacillus* species (*B. anthracis, B. cereus and B. subtilis*) into culture media were prepared by successive steps of inoculation of the culture media with spores, overnight incubation at 37°C, removal of bacterial cells by centrifugation, sterilization of the supernatant by filtration through 0.22 μ filter and further 50-fold concentration using ultrafiltration devices Amicon Ultra 15 (Millipore, MA) with a 10 KDa cutoff size. The SDS-PAGE gel separation (Fig. 2.1A) demonstrates the protein content in the concentrated *B. anthracis* culture supernatant (designated as BACS) used in our animal tests. Similar procedures were used to prepare culture supernatants for *B. cereus* ATCC #11778 and *B. subtilis* ATCC #23857 (designated BCCS and BSCS, respectively).

The concentrated culture supernatants were tested in mice. Upon subcutaneous administration, mice developed hemorrhages of different intensity within several hours (Fig. 2.2A, B). BCCS showed the highest activity followed by BACS, while BSCS was completely inactive. Chemical inhibitors such as phosphoramidon (potent chelating inhibitor of thermolysin and other M4 bacterial metallo-endopeptidases , Komiyama *et al.*, 1975), EDTA (specific for a broad range of MPs) and soybean trypsin inhibitor (SBTI, reversible competitive inhibitor of trypsin and other trypsin-like proteases such

as chymotrypsin, plasmin and plasma kallikrein) effectively abrogated the hemorrhagic affect of BACS (Fig.2.1C).

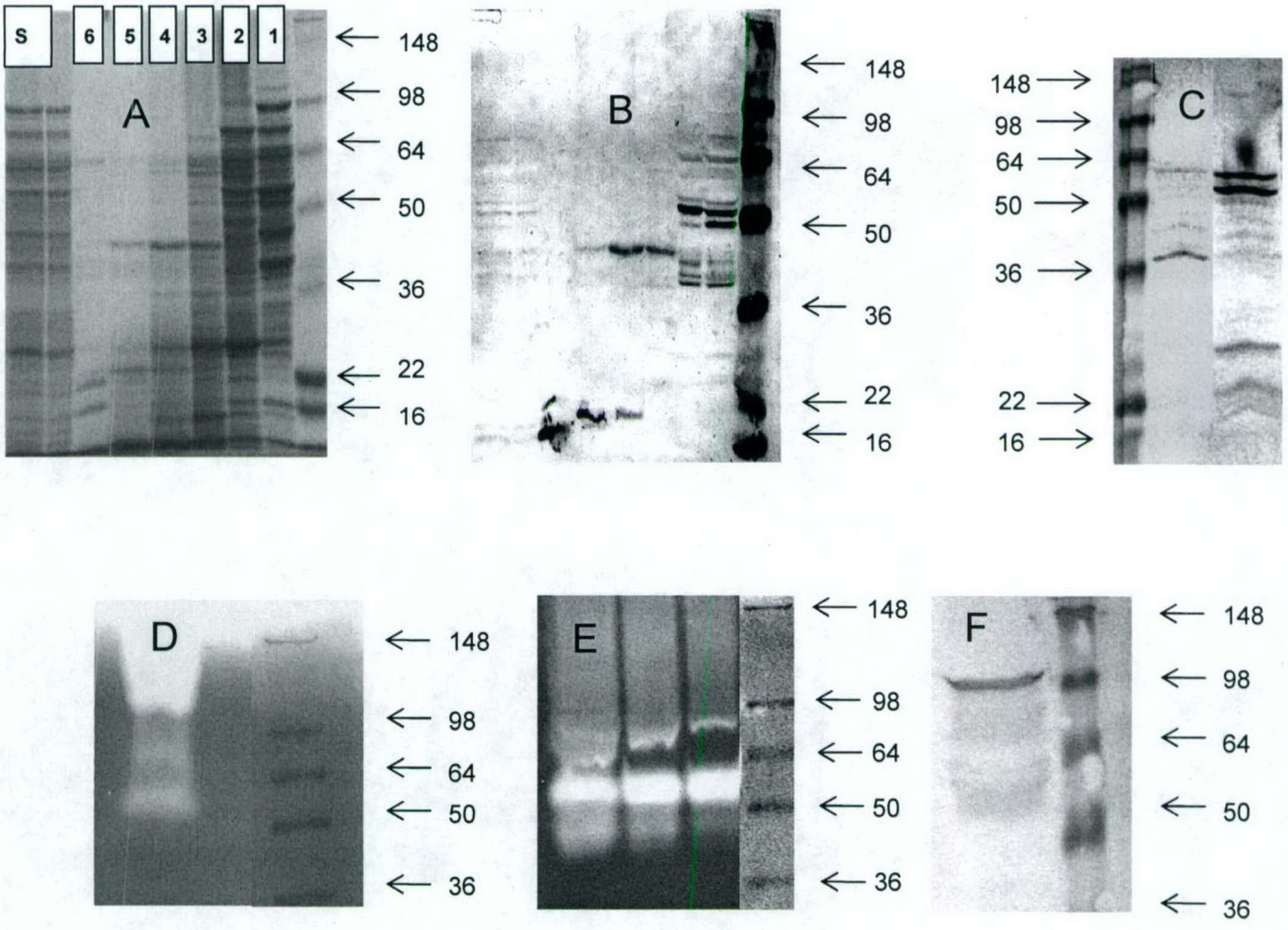


Fig. 2.1. SDS-PAGE of BACS fractions separated on size exclusion column (A), Western blots: fractions (A) with specific antisera a-M4EL (B), BACS with a-M4AC (C, left panel), BACS with a-M4EP (C, right panel), BACS with a-M9Coll (F), and zymograms of caseinolytic (D) and gelatinolytic (E) activities of BACS. Molecular masses (KDa) of the marker proteins are indicated by arrows. In A, s denoted BACS, and numbers above correspond to column fractions. In E, different amounts of BACS were loaded on a gel (15 μl, 7 μl and 3 μl, from left to right).

The murine serum raised against the recombinant protein corresponding to the mature form of the M4-type thermolysin-like neutral protease of *B.anthracis* (BA 3442) was also effective in suppressing the hemorrhagic effect in the skin test. In negative control experiments, neither naïve murine serum nor three irrelevant murine sera against *B. anthracis* hemolysins O, A and B (Klichko *et al.*, 2003) showed anti-hemorrhagic activity (data not shown). Additional control experiments demonstrated that under the conditions of our test the hemorrhagic activity of thermolysin from *B. thermophilicus* was detectable in a dose range from 10 to 100 μg, similar to that for BACS.

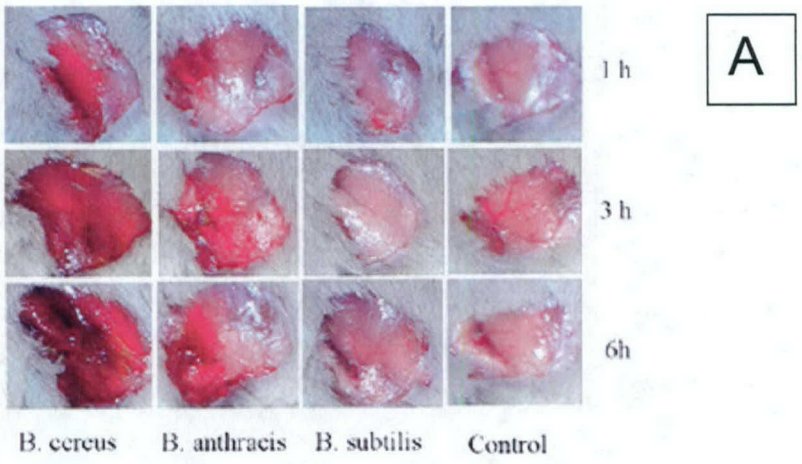


Fig. 2.2. Hemorrhagic activity of culture supernatants (A), its graphic representation (B) and inhibition with chemical inhibitors (C).

In contrast to BACS, the inhibitors displayed only partial protection in the case of BCCS (Fig.2.2C). Overall, these results indicated that a hemorrhagic activity in BACS was represented by a single or possibly a small number of enzymes of the MP-type, while BSCS contained a more heterogeneous array of enzyme activities. This conclusion is consistent with the experimental data that *B. anthracis* culture supernatants are less toxic to mice compared to *B. cereus* ones (Bonventre *et al.*, 1963; Ezepchuk *et al.*, 1969).

Caseinase and gelatinase activities of BACS are readily detected by zymography using casein or gelatin (denatured collagen) (Fig.2.1D, E). A major band of gelatinase activity

corresponds to molecular mass of about 100 KDa, whereas a collagenase activity is represented by about 55 KDa proteins.

Generation of antibodies against B. anthracis MPs. Obvious complexity of the BACS protein composition prompted us to develop specific means of detection and inhibition of its components. For this purpose several immune sera were raised in mice and rabbits using the antigens listed in Table 2.1.

Table 2.1. Sera against *B. anthracis* proteases

Serum #	Protein family	Protein	Gene number	Antigen	Designation
1	M4	Elastase-like neutral protease	BA3442	Recombinant polypeptide corresponding to the fragment 248-532.	M4EL
2	M9	Collagenase	BA0555, BA3299, BA3584	HEFTHYLQGRYEVPGL spanning the region of active center	M9Coll
3	M4	Neutral protease	BA5282, BA0599	DVIGHELTHAVTE spanning the region of active center	M4AC
4	M4	Neutral protease	BA2730	ADYTRGQGIETY distant from the active center	M4EP

The sera were used in Western blots of BACS proteins. When the proteins were directly separated in the SDS-PAGE for subsequent transfer to the nitrocellulose membrane, the resulting blots were of low intensity indicative of proteolytic degradation during the electrophoresis (Fig.2.1A, left lane). In order to avoid this complication the BACS was fractionated according to the molecular masses of its components on the Superdex size exclusion column in the presence of EDTA as a chelating agent. Analysis of the column fractions in SDS-PAGE showed a complex pattern of proteins bands (Fig. 2.1). Multiple proteins with a broad spectrum of molecular masses seem to be highly associated and migrate through the column as high molecular mass complexes. Several factors, such as the presence of multiple precursor and mature protein forms resulting from specific proteolytic maturation, along with nonspecific proteolytic products, can potentially contribute to the complexity of the fractions' composition. Western blot experiments with column fractions revealed several discrete bands recognized by antibodies (Fig. 2.1). The M4 proteases are represented by several bands at about 50 KDa, as well as by the bands at about 40 and 20 KDa. These bands probably correspond to different maturation forms of proteases, including the enzymes lacking signal peptides, and mature enzyme forms. The M9 collagenases are detected as a band with a

molecular mass of about 98 kDa which is close to the estimated mass of the pro-enzymes, however the major gelatinase enzymatic activity corresponds to the 55 kDa proteins in the BACS.

Acute toxicity of B. anthracis culture supernatants. Although bacterial proteases are well known pathogenic factors, little information is available regarding their acute toxicity. We tested BACS in mice using intratracheal administration into the lungs because hemorrhagic mediastinitis and lung edema typically precede the lethal outcome in late anthrax. Therefore, lung damage may be considered as a probable death-causing factor. Mice were given different doses of BACS (10 μg to 40 μg of total protein) and were observed daily for lethality. Fig. 2.3 shows that depending on the dose all mice died within 2 to 3 days, while the highest dose caused 80% mortality on day 1. For histopathological examination mice were given 100 μg of BACS protein.

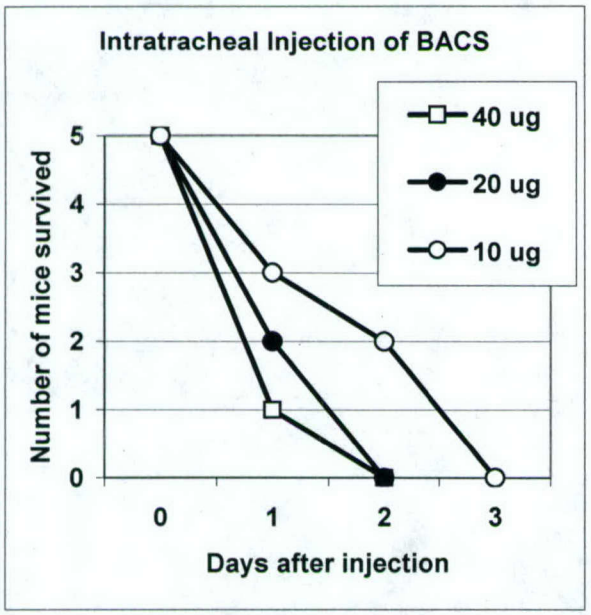


Fig.2.3. Survival of mice upon intratracheal injection of BACS.

All animals died within 3 to 4 hours. Postmortem harvested lungs revealed focal intraalveolar acute hemorrhage, which was from minimal to moderately severe with no endothelial cell damage or vasculitis, and mild patchy congestion of medium-size blood vessels. There was evidence of focal platelet accumulation located within areas of hemorrhage or within vessels. In a control experiment, lethal toxin at a comparable dose (100 μg LF, 100 μg PA) caused neither mortality nor hemorrhage, and in fact, produced no significant identifiable histopathological changes.

Protection of mice against anthrax using protease inhibitors. [1] Effective suppression of the hemorrhagic as well as the proteolytic activity of BACS with chemical inhibitors prompted us to test their protective effect against *B. anthracis* infection. We have previously reported the successful application of an adjunct therapy against anthrax infection targeting both bacterial multiplication and host response to infection by using a combination of antibiotic and caspase inhibitors (Popov *et al.*, 2004). The same principle was used in the current study because inhibition of secreted pathogenic factors is not expected to directly interfere with bacterial multiplication and therefore may not be

[1] These experiments were carried out in order to obtain initial proof-of-principle data. We currently continue animal protection studies under another project.

fully protective. In order to target both the bacteria and the proteolytic factors we used a combination therapy where antibiotic administration was complemented by the administration of a protease inhibitor. We were also interested in the efficacy of delayed treatment initiated after certain periods of time following spore challenge. It is a practically relevant scenario because patients generally seek medical help after the onset of symptoms, and in other patients treatment begins after a certain period of time required to confirm the exposure. In addition, a delayed ciprofloxacin therapy in murine model is only partially protective when currently recommended human antibiotic doses (adjusted for body weight) are used in mice (Popov *et al.*, 2004). It seemed interesting to study if a combination approach could lead to a synergistic enhancement in survival. Two chemical inhibitors were chosen for this study, phosphoramidon and phenanthroline. Phosphoramidon is a potent inhibitor of thermolysin and other bacterial metallo-endopeptidases, and is effective in suppressing the hemorrhagic effect of BACS. It does not inhibit trypsin, papain, chymotrypsin or pepsin and weakly inhibits collagenase. Phenanthroline is a potent chelating inhibitor of M4 MPs, such as pseudolysin, as well as matrix MPs (Supuran *et al.*, 2002).

Results of three independent experiments are presented in Figs. 2.4 and 2.5. Mice were challenged intraperotoneally (i.p.) with about $1x10^7$ of B. anthracis Sterne spores. Treatment with a single daily dose of ciprofloxacin (50 mg/kg, i.p.) began immediately after challenge, as well as at 24 h or 48 h post challenge, and continued for 10 days. In our conditions the ciprofloxacin treatment initiated immediately after spore challenge was only 70% effective in preventing death. The survival rate after a 24 h delay in antibiotic administration produced a sharp decline to 20% but remained statistically reliable (compared to untreated group, $p=0.015$). After a 48 h delay the antibiotic was completely ineffective ($p=0.23$). The inhibitor treatment without antibiotic was not able to improve survival, however the combination of ciprofloxacin with inhibitors displayed a synergistic increase in protection, especially notable in the case of phenanthroline. The group receiving phenanthroline/ciprofloxacin treatment delayed by 24 h, demonstrated 70% protection of animals, compared to only a 20% survival in the group with ciprofloxacin alone ($p=0.03$ for these groups). The 48 h-delayed regimen resulted in a statistically reliable 30% protection (relative to the untreated spore-challenged group, $p<0.05$), in contrast to ciprofloxacin alone (relative to the untreated spore-challenged group, $p=0.23$). There is a similar trend in the efficacy of the combination phosporamidon/ciprifloxacin therapy, compared to ciprofloxacin alone, however the observed differences are less reliable ($p>0.05$).

***Protection of mice against B. anthracis using anti-protease sera*[2]**

Neutralizing activity of antibodies against proteases in vitro as well as in vivo prompted us to test their protective effect against B. anthracis infection (Fig. 2.6). As in the experiments with

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[2] These experiments were carried out under this project in order to obtain initial proof-of-principle data. We currently continue animal protection studies under another project.

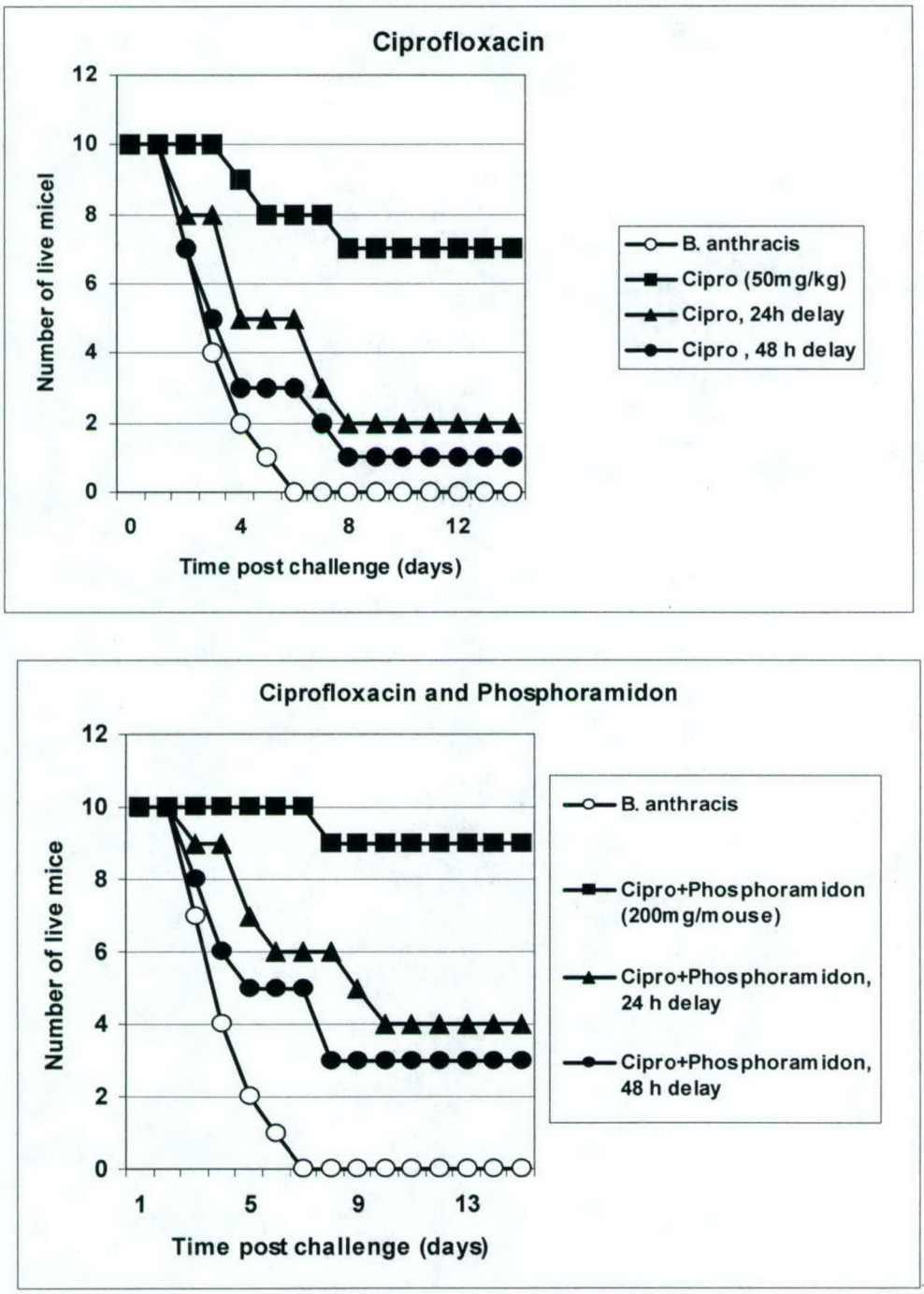
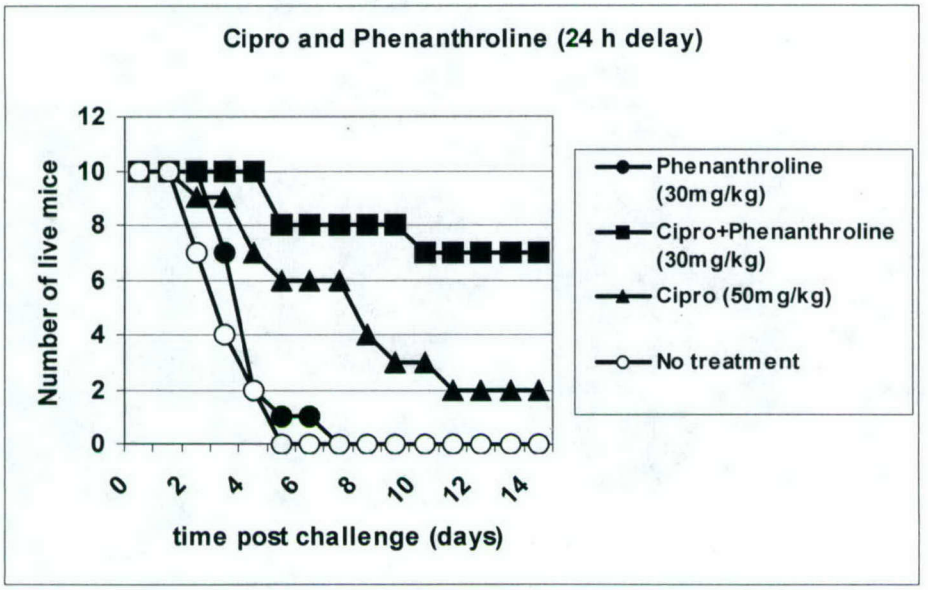


Fig. 2.4. Protection of mice against *B. anthracis* (Sterne) infection by administration of ciprofloxacin and its combination with phosphoramidon for 10 days beginning 24 h and 48 h post spore challenge.



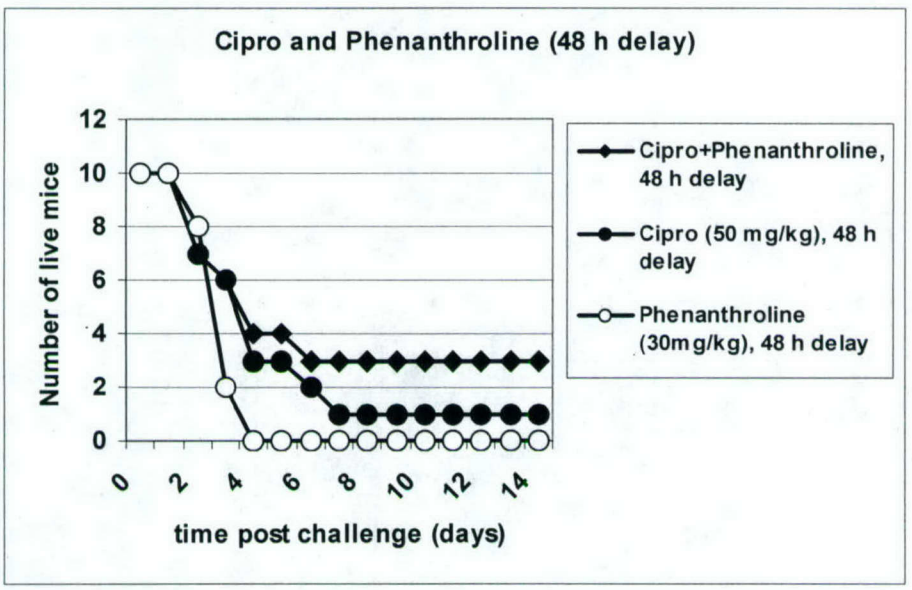
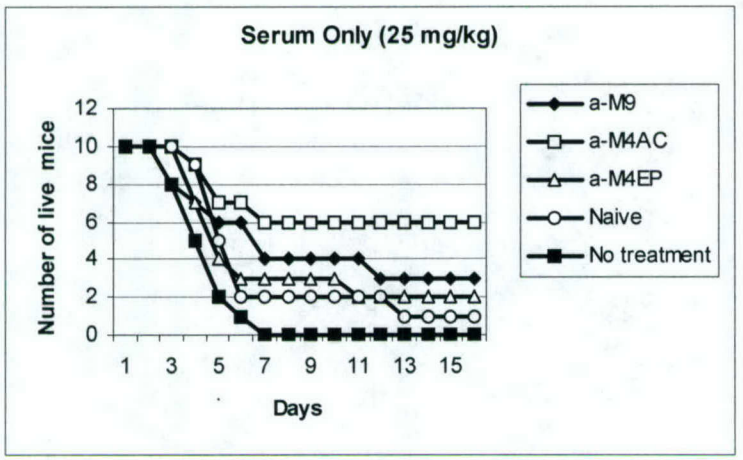
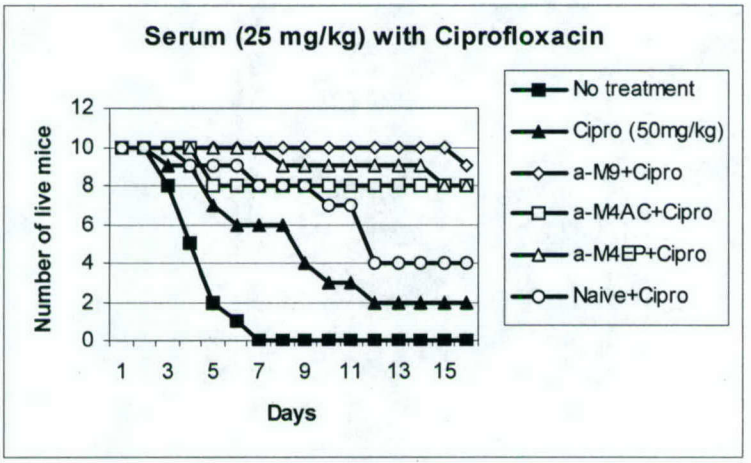


Fig. 2.5. Protection of mice against *B. anthracis* (Sterne) infection by administration of ciprofloxacin or its combination with phenanthroline for 10 days beginning 24 h and 48 h post spore challenge.





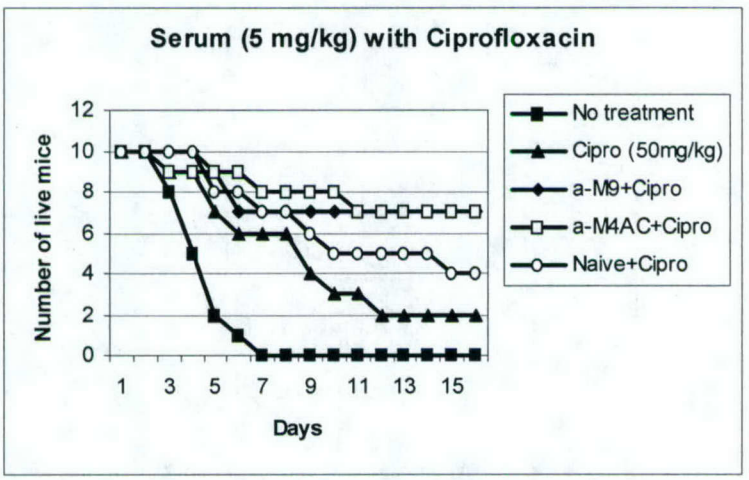


Fig.2.6. Post exposure efficacy of hyperimmune rabbit sera in mice challenged with *B. anthracis* (Sterne). Treatment with sera and ciprofloxacine was initiated 24 h post exposure and continued for 10 days once daily.

inhibitors, mice were challenged intraperitoneally (i.p.) with about 30 LD50 of B. anthracis Sterne spores. Treatment with a single daily dose of ciprofloxacin (50 mg/kg, i.p.) began at 24 h post challenge and continued for 10 days. The immune sera (each pulled from two rabbits) were administered once daily at a concentration of 25 mg/ml (i.p.). The sera displayed substantial differences in their protective effect. The anti-M4 serum against the epitope(s) of the active center displayed the highest protection (60%), while the anti-collagenase serum (a-M9Coll) protected 30% mice. The anti-M4EP serum behaved similar to the naïve serum. Both latter sera demonstrated no statistically reliable difference in survival, compared to untreated mice (10%, $p>0.05$). A combination treatment with both antibiotic and all studied immune sera, administered at the same dose (25 mg/kg) was synergistic and protected from 80 to 100% mice. A lower serum dose (5 mg/kg) showed a similar pattern of protection, however the effect of combination treatment was reduced to 70%.

2.3. Discussion and Conclusions

In order to be highly virulent, any pathogenic microbe is required to possess the means to effectively establish and further propagate the infectious process. Distinct virulence factors may be necessary to fulfill these requirements at different stages of the disease. *B. anthracis* is a recently emerged highly virulent pathogen which acquired two plasmids pXO1 and pXO2 compared to the genetically similar but opportunistic pathogen B. cereus. These plasmids encode for the lethal toxin (LT) and capsule genes, respectively (Inglesby *et al.*, 2002). Historically, LT was the first anthrax virulence factor discovered capable of causing death in experimental animals (Smith and Keppie,1954). This property of LT essentially abrogated further research efforts on discovery of other potential virulence factors, however it has long been known and now seems well established that LT is not especially toxic (Smith *et al.*, 1955; Friedlander, 2001), and that the histopathology of LT intoxication differs considerably form that found in clinical anthrax infection (Moayeri *et al.*, 2003; Cui *et al.*, 2004). It is especially notable that postmortum examination of victims from the Sverdlovsk accident and those autopsied following the 2001 U.S. anthrax attacks, revealed hemorrhagic thoracic lymphadenitis and necrotizing hemorrhagic mediastinitis in all patients. About half of the Sverdlovsk victims additionally had hemorrhagic meningitis (Grinberg *et al*, 2001). These severe life-critical symptoms had not been noticed in the intoxicated animals (Moayeri *et al.*, 2003; Cui *et al.*, 2004). Recent data, however, implicate a new role of LT as a disease-establishing virulence factor playing an important immunosuppressive role within alveolar macrophages at the early stages of inhalation anthrax (Pellizzari *et al.*, 1999). It has been demonstrated that LT causes apoptotic death of macrophages and its inhibition decreased survival of *B. anthracis* spores engulfed by macrophages (Popov *et al.*, 2002a,b). These data suggest that the major rolls of LT and ET may actually be to create a more immunologically hospitable environment for the pathogen.

While only fragmented data have been reported on the existence of *B. anthracis* chromosome-encoded virulence factors (Mikshis *et al.,*1999, Stepanov *et al.*, 1999; Klichko *et al.*, 2003), it is well established that *B.cereus* produces a variety of pathogenic determinants, including a necrotizing enterotoxin, an emetic toxin, extracellular proteases, phospholipases and hemolysins (Dobriniewski, 1993). B. cereus is capable of causing serious and sometimes lethal infections such as sepsis, pneumonia, meningitis, endocarditis, wound and ocular infections, especially in immunocompromized individuals (Akesson *et al.,* 1991; David *et al.*, 1994; Turnbull *et al.*, 1977; Drobniewcski, 1993; Helgason *et al.*, 2000). A highly virulent isolate of B. cereus has recently been identified which contains a plasmid 99.6% similar to pXO1. This finding is consistent with the point of view that the B. cereus genetic background is sufficient for high virulence when it is complemented with an infection-establishing virulence factor, such as LT. Complete sequencing of the B. anthracis and B. cereus genomes confirm their close relationship suggested previously (Helgason *et al.,* 2000) and allowed us to suggest new candidate virulence factors for B. anthracis, specifically the proteases of the M4 and M9 families. Structurally similar proteolytic factors in other pathogenic microorganisms are known to be involved in inactivation of complement factors (Clearly *et al.*, 1992), cleavage of serum protease inhibitors (Ishihara *et al.,* 1996*)*, activation of blood coagulation system (Kaminishi *et al.*, 1994), invasiveness into the host tissue (Sodeinde *et al.*, 1992), and development of hemorrhages (Miyoshi *et al.*, 1998).

We demonstrate here that secreted metalloproteases (MPs) of B. anthracis can digest protein substrates such as casein and gelatin in vitro, and can induce a hemorrhagic process in our test subjects, in vivo. Both of these activities are inhibited by chemical inhibitors of M4 and M9 MPs, such as EDTA, phosphoramidon and 1,10-phenanthroline. Consistent with this, the hyper-immune mouse serum against M4 family thermolysin/elastase-like enzyme is capable of inhibiting the hemorrhagic effect of BACS (we currently investigate the *in vitro* comparative inhibiting activity of the hyper-immune sera used in this report). The tissue-damaging action of this type of enzyme is well known (Supuran *et al.,* 2002). For example, pseudolysin, an elastase of Pseudomonas aeruginosa, destroys arterial elastic laminae in systemic infection (Kon *et al.,* 1999), causes lung damage with hemorrhages and necrosis, causes destruction of epithelial cell function, and induces septic shock through activation of the host kinin cascade.

In the present study we used an intratracheal (i.t.) administration to demonstrate that tissue destructive and hemorrhagic properties of BACS could cause a lethal effect at 0.5 to 3 mg/kg doses (10 to 60 μg per 20g DBA/2 mouse) within a few days or even hours (Fig. 3). Histopathological examination confirmed our observations of life-threatening severe bleeding upon administration of BACS. It has been reported that *P. aeruginosa* elastase induced an immediate lethal shock in guinea pigs upon an i.v. injection at a similar dose of 1.2 mg/kg. Compared to BACS, the LT is non-toxic upon an i.t. administration. There was no mortality in a control group of LT-treated mice (200 μg/mouse, i.t.). Recently, the toxicity of highly purified LT was re-evaluated in BALB/CJ and

C57BL/6J mice, and it was found that doses from 5 to 12.5 mg/kg (i.v.) were required for up to 90% mortality in 5 days (Moayeri *et al.*, 2003). We conclude that secreted proteins of B. anthracis, in addition to LT and ET, have high pathogenic potential and should be considered as important virulence factors.

We have previously suggested a combination antibiotic-antitoxin approach to anthrax therapy and for the first time demonstrated an increased efficacy of ciprofloxacin treatment in a murine model when it was combined with a caspase inhibitor administration (Popov *et al.*, 2004). Up to now, there has been no other report on any anti-LT treatment during the anthrax infectious process. In the present study we used two new combination therapies, namely the antibiotic-protease inhibitor and the antibiotic-antiserum ones. Both of them also proved beneficial, compared to antibiotic alone. The inhibitors we used to model anthrax therapy (phosphoramidon and phenanthroline) are not considered as LT inhibitors, however both of these substances increased survival during ciprofloxacin therapy, which was initiated at 24 h and 48 h post challenge. The phenanthroline-ciprofloxacin combination administered 24 h post challenge protected 70% of the mice, compared to 20% for antibiotic alone. Late stages of anthrax are especially difficult to treat (Inglesby *et al.*, 2002). In our model the 48 h post challenge time approximately correlates with the period of typical overt anthrax symptoms in patients because 30% of mice die within the next 24 h, a situation that is similar to that clinically observed in natural human infection. In these circumstances our therapy with phenanthroline-ciprofloxacin was 30% protective, while ciprofloxacin alone was ineffective. It is worth pointing out that the high extent of protection (70 after 24 h delay) conferred by a combination of antibiotic with phenanthroline argues against the predominant role of LT as a death-causing factor. We have previously reported that in similar experimental conditions the caspase inhibitor YVAD, capable of protecting macrophages against LT-induced apoptosis, improved survival of DBA/2 mice by 30%. This figure corresponds well to the maximal expected contribution of LT to overall lethality based on our current data. One may therefore expect that a triple component therapy, such as ciprofloxacin-phosphoramidon-caspase inhibitor, might be completely protective. Experiments in this direction are currently in progress.

Overall our data demonstrate that B. anthracis cultivated in culture media secrets a number of proteolytic virulence factors, including those with hemorrhagic, caseinolytic and gelatinolytic activities. These factors in most respects are distinct from LT, including the mode of their expression under aerobic conditions (LT requires bicarbonate for its expression in vitro [Wright *et al.*, 1954]), their molecular targets, as well as a high virulent potency upon intratracheal administration. Chemical inhibitors of these factors demonstrate a substantial protective efficacy in combination with antibiotic therapy. Our findings outline a new direction in the development of anthrax therapeutic approaches, and close a substantial gap between the understanding of anthrax molecular pathology and the most prominent clinical features of its infectious process. Complexity of the BACS composition with regard to the number and specificity of proteolytic enzymes suggests

a multitude of their potential virulent mechanisms that need to be explored further. Part of this data has been submitted for publication in the journal Antimicrobial Agents and Chemotherapy.

2.4. Materials and Methods

Microbial strains. The non-encapsulated *Bacillus anthracis* strain 34F2 (Sterne) [pXO1+, pXO2-] obtained from the Colorado Serum Company (Boulder, CO) was used in animal challenge experiments. The 50% lethal doses (LD_{50}s) by the inraperitoneal (i.p.) route were established earlier (Popov *et al.*, 2004) and the LD_{50} value for intraperitoneal challenge for DBA2 mice was found to be 3×10^6 spores per mouse. The non-encapsulated, atoxigenic strain of *B.anthracis* (delta Ames) [pXO1-, pXO2-] was kindly provided by Dr. J. Shiloach (National Institutes of Health, Bethesda, MD). *B. cereus* strain ATCC #11778 and *B. subtilis* strain #23857 were purchased from American Type Culture Collection (Manassas, VA).

Mice. Female DBA2 mice (9 weeks old) were obtained from Taconic (Germantown, NY) and were used throughout the study.

Reagents. The following substances were used in this study: ciprofloxacin (ICN Biomedicals, lot no.4913F), phosphoramidon disodium salt, and 1,10-phenanthroline (Sigma, MO), EDTA (GibcoBRL, CA), soybean trypsin inhibitor from Glycine max (Sigma, MO), thermolysin (EC 3.4.24.27) from *Bacillus thermoproteolyticus* (Sigma, MO). The fluorescently labeled casein and collagen type I for determination of proteolytic acivity were from Molecular Probes (OR). Zymogram gels were from Invitrogen (Carlsbad, CA). Lethal factor (LF) and protective antigen (PA) were from List Biological Laboratories (CA).

Preparation of secreted proteins. Secreted substances were prepared by culturing *B. anthracis* (delta Ames) in LB media overnight. Cells were removed by centrifugation at 8000 g, and the supernatant was sterilized by filtration through 0.22 μm cellulose acetate filtration system (Corning, NY) and further concentrated 50-fold using Amicon Ultra15 centrifugal filter devices (10K cut-off pore size) (Millipore, MA). The proteins were used immediately after preparation or were stored at 4°C for several days. Protein content was determined using Bradford reagent (Bio-Rad) with bovine serum albumin as standard. Slow reduction in the hemorrhagic activity was found upon storage within a week.

Fractionation of culture supernatants. 1 ml of *B.anthracis* culture supernatant (BACS) was loaded onto the size-exclusion Superdex column (25x60, Pharmacia Biotech) and was eluted with PBS (pH 7.4) with a flow rate of 2 ml/min. Fractions of eluate were concentrated to equal volumes using Centricon devices (Millipore, MA) with a 10K cut-off pore size.

Hemorrhages in femoral artery region. Mice were anesthetized by intraperitoneal injection of Avertin (2,2,2 tribromethanol, Aldrich) and 100μl of *B.anthracis* secreted proteins (20 to 100 μg) were subcutaneously (sc) injected into the femoral artery region for observation of hemorrhagic changes after 3 to 15 h. In order to record hemorrhagic changes animals were anesthetized by i.p.

injection of Avertin and the fur over the femoral artery region was removed to allow open observation of a 1.5 to 2.5 cm^2 area of skin. It was photographed, and the size of the hemorrhagic spot was measured. In the experiments on the inhibition of hemorrhagic effect the secreted proteins were preincubated with specific antisera or protease inhibitors for 30 min on ice.

Generation of antibodies against B. anthracis MPs. The Invitrogen (CA) custom service was used to obtain rabbit polyclonal sera against peptides conjugated with kallikrein (Table 1). Two animals were immunized by each conjugate. All six rabbit sera had ELISA titers ranging from 100,000 to 200,000. For generation of murine polyclonal antibodies against the M4 protease (BA3442) the C-terminal part of the gene encoding amino acids 248 to 532 was cloned into pTrcHis2 TOPO TA cloning vector (Invitrogen, CA). The recombinant protein containing a 6xHis tag was expressed in *E.coli* and purified using the Ni-NTA resin (Quiagen, CA). Mice were immunized with 50 μg of the protein emulsified in a complete Freund's adjuvant and were given two booster immunizations using an incomplete adjuvant with 2 week intervals. Serum was collected after two weeks since the last boost injection. In the skin hemorrhagic test described above, 30 μl of serum were able to completely suppress the hemorrhage caused by 30 μl of BACS.

Intratracheal delivery of B. anthracis secreted proteins. Mice were anesthetized by i.p. injection of Avertin and a 24G angiogenic catheter (BD Biosciences, CA) was inserted into the trachea. 50μl of experimental mixture, containing 10 to 100 μg of culture supernatant proteins were slowly injected through the catheter connected to a microsyringe. The angiogenic catheter was removed and animals were left for further observation. The untreated control group received the same volume of phosphate-buffered saline (PBS). A control group of 3 animals was injected with 50 μl of PBS solution of lethal toxin (100μg PA+100μg LF). In all experiments the rate of breathing was recorded every 10 min during the first 3 h following injection, and animals were observed for survival for 7 days.

Treatment of spore-challenged mice. Mice used in all experiments were maintained under proper conditions with a 12-h light/dark cycle in accord with IACUC standards in the animal facility of the Biocon, Inc. (Rockville, MD). Mice received food and water *ad libitum.* Groups of 10 mice were randomly assigned for challenge and were observed for survival and signs of disease. The animals were inoculated i.p. by 1×10^7 spores per mouse of Sterne strain. Treatment (i.p.) with phenanthroline (30 mg/kg), phosphoramidon (10 mg/kg), or rabbit sera (5 or 25 mg/kg) was carried out individually for each substance or in combination with ciprofloxacin at (50mg/kg) once a day started at different time points post spore challenge and continued for 10 days. In all experiments the animals were monitored for survival for at least 12 days after termination of treatment.

Statistical analysis. Kaplain-Meier open-end survival analysis was performed to compare results between treatment groups. Statistical significance was established as $P<0.05$ using log-rank test.

TASK 3. TOLL-LIKE RECEPTOR (TLR)-NEUTRALIZING ANTIBODIES AND SOLUBLE TLRS AS SPECIFIC AND BROAD-SPECTRUM PROTECTION AGAINST BIOLOGICAL WEAPONS

3.1. Background

Toll-like receptors have been considered sensors of infection and can induce the activation of innate and adaptive immune responses against invading pathogens. Therefore, TLRs are receiving considerable attention as regulators and controllers of the immune response through their ability to recognize pathogen-associated molecular patterns (PAMP). The interaction of TLRs with infectious pathogens including bacteria, fungi, and viruses has been broadly established (Fig. 3.1) but has not been thoroughly studied.

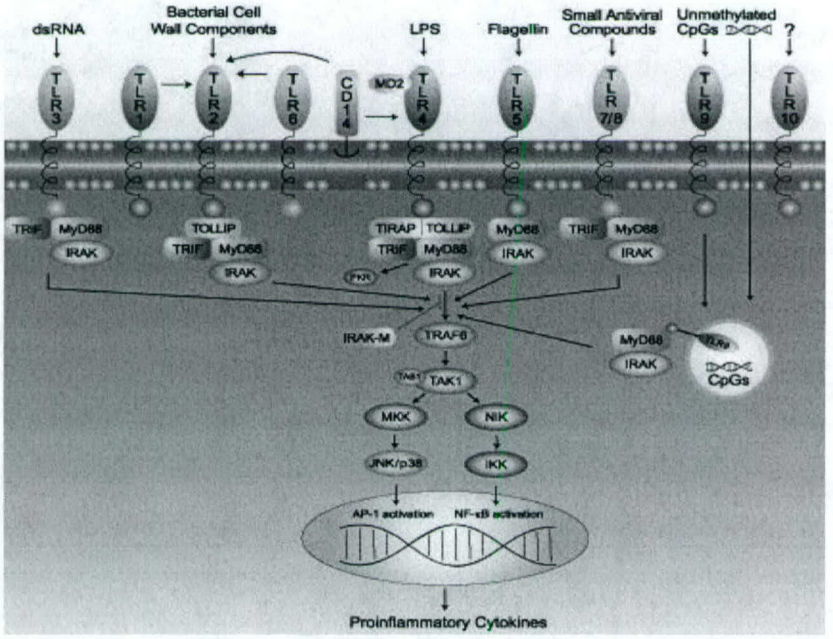


Fig. 3.1.1. Diagram of TLRs and their ligands. Ten human TLRs and seven of their ligands have been identified. Stimulation of TLRs will trigger a common signaling cascade and results in the production of proinflammatory cytokines.

As first models of study we use *B. anthracis* and VV infections. There are striking differences in the lethality of anthrax infection exhibited in various mouse strains. A remarkable paradigm is that inbred mice strains C3H/HeN and C3H/HeJ have similar genetic backgrounds except that C3H/HeJ mice have a point mutation in the TLR-4. While C3H/HeN is sensitive to Sterne strain of *B. anthracis*, C3H/HeJ is more resistant (Welkos et al., 1986). It is possible that *B. anthracis* can be detected by TLR-4 and that the TLR-4 deficiency impairs the macrophage response (Kim et al., 2002). Whether this is due to genetic differences or variance in the number of TLRs on macrophages remains unknown. It is also well known that human macrophages, as well as

macrophages from a variety of mouse strains, are insensitive to anthrax lethal toxin (LeTx)-induced apoptosis. It has recently been demonstrated that macrophage susceptibility to LeTx can be increased by stress signal (Popov *et al.,* 2002b) or stimulation with innate antigens (Park *et al.,* 2002). It is now established that the classical macrophage activation involves TLR receptors, which play a key role in the inflammatory response and apoptosis (Blander and Medzinov, 2004).

Our preliminary studies showed that anthrax lethal toxin (LeTx) is a potent inducer for histamine release from human basophils (Wu *et al.,* 2003a). This effect could be completely eliminated in the presence of antibody against TLR-2. The phagocytic engulfment of *B. anthracis* Sterne spores by human macrophages was impaired when TLR-4 antibody was added.

Recently, the roles of TLRs in the recognition of viral products from HIV, type I Lang retrovirus, respiratory syncytial virus, and mouse mammary tumor virus (MMTV) have been clarified (Otten *et al.,* 2002). Once again the TLR-4 deficient strain C3H/HeJ holds a low incidence of tumors induced by MMTV infection compared to the C3H/HeN strain. Poxvirus family of DNA viruses includes two closely related species, namely variola and vaccinia (VV) viruses. VV displays a unique ability to evade the host immune responses, such as producion of secreted decoy receptors for cytokines including IL-1, TNF-α, chemokines, INF-α/β and INF-γ (Alcami *et al.,* 1997, 1998; Smith *et al.,* 1997; Liptakova *et al.,* 1997). Internalization of biological agents is a critical step to initiate infection. It is plausible that TLRs play a role in poxvirus pathogenesis such as inflammation and the internalization of invading pathogens and it may be another avenue for the development of novel prophylactics and treatments.

3.2. Results.

3.2.1. Effect Of Anti-TLR Antibodies On Macrophages, Anthrax Spores And Intracellular Proliferation Of Vegetative Bacilli In Macrophages

Antibody against TLR4 blocks inflammatory cytokine production induced by anthrax protective antigen (PA). PA is a moderate inducer for cytokine production by human peripheral blood mononuclear cells (PBMCs) as we tested before. We further tested the blocking effects of neutralizing antibodies against TLR2 and TLR4 on inflammatory cytokine production. Three healthy donors participated in this experiment. PBMCs were treated with the antibodies for 30 min at 37°C, and PA was introduced into the culture thereafter. The culture incubated at 37 °C for 24 h. Supernatants from the culture were harvested. The concentration of various cytokines was measured using flow cytometry after staining with CBA kit produced by BD-Pharmingen.

The CBA kit can simultaneously test 5 inflammatory cytokines that include IL-8, IL-10, TNF-α, IL-6, and IL-1β. PA alone could induce the production of the cytokines. However, this effect was removed in the presence of antibody against TLR-4. Surprisingly, antibody against TLR-2 had

synergetic effect with PA in inducing cytokine production. The amount of cytokines in LeTx-treated group was the lowest that is consistent with our previous finding. The following figures represent the average concentrations from three different donors.

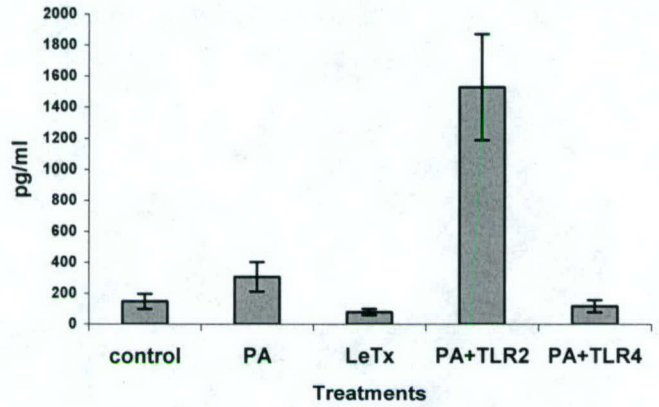


Fig. 3.2.1. IL-6 production by human PBMC after treatment with PA, LeTx, and antibodies against TLRs

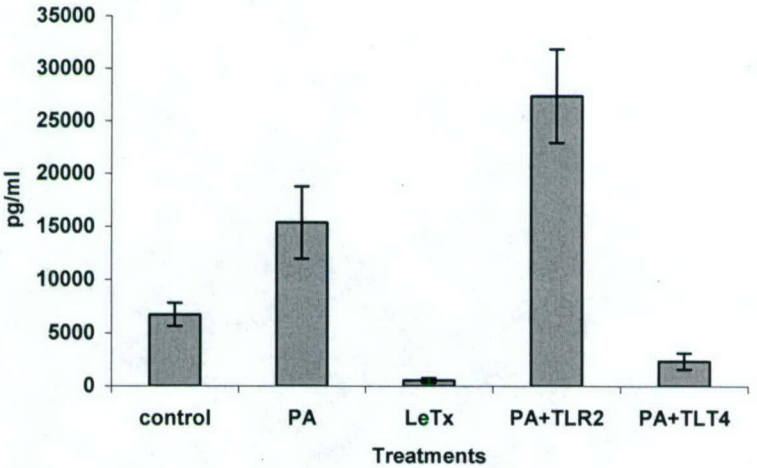


Fig.3.2.2. TNF-α production by human PBMC after treatment with PA, LeTx and TLR antibodies

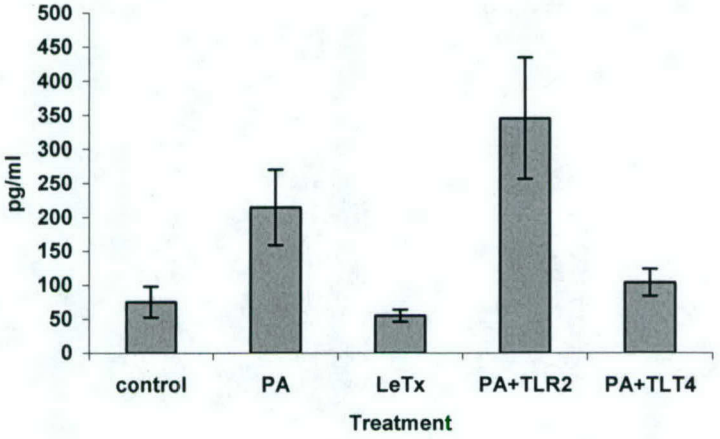


Fig.3.2.3. IL-10 production by human PBMC after treatment with PA, LeTx, and TLR antibodies

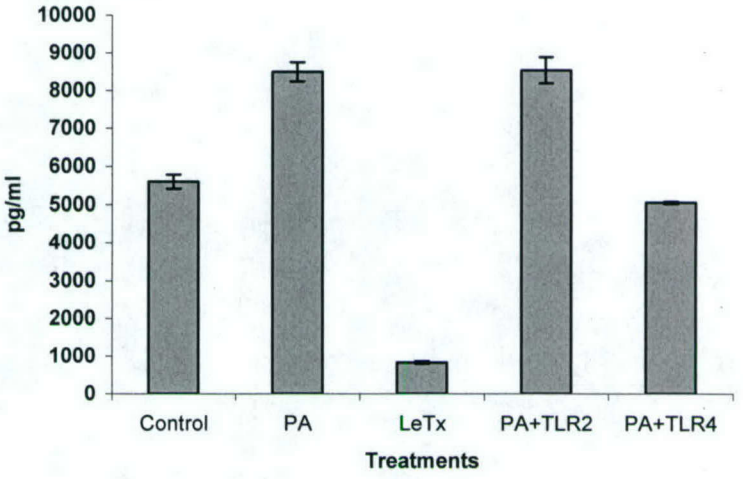


Fig. 3.2.4. IL-1β production by human PBMC after treatment with PA, LeTx, and antibodies against TLRs

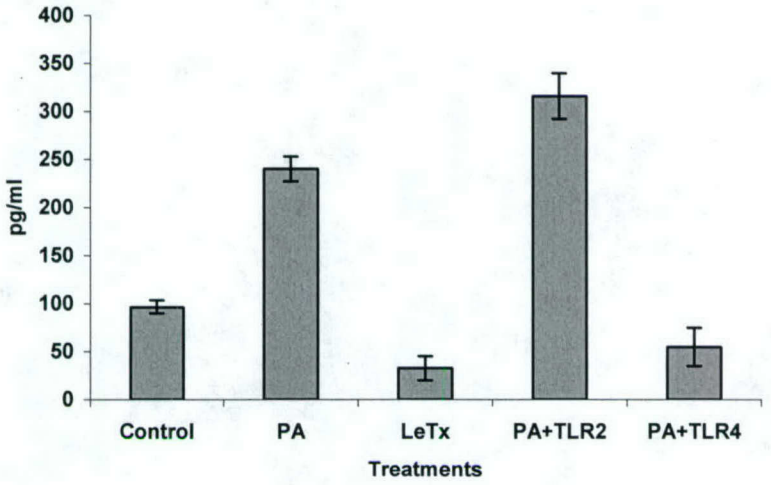


Fig. 3.2.5. IL-8 production by human PBMC after treatment with PA, LeTx, and antibodies against TLRs

PA binding to CHOK1 and CHOR1.1 cells transfected with hTLR2 and hTLR4 expression constructs. The CHOK1 cell line is a Chinese hamster ovary cell line, which does not express TLRs. The CHOR1.1 cell line is a CHOK1 mutant cell line created by Dr. Bradley (UCLA, CA), which lacks the PA receptor. These cell lines are useful reagents to test possible interactions between TLR2/4 and PA. In our previous studies a blocking antibody against TLR2 was able to inhibit lethal toxin induced histamine release in PBMCs from human donors suggesting a possible link between TLR2 and lethal toxin. Because PA is critical for lethal factor's entry into cells, we were interested in focusing on PA-TLR binding studies. To test the ability of the PA to bind to TLRs, FITC-labeled protective antigen from *B. anthracis* was used to test whether PA would bind to TLR2 or TLR4 transfected cells using flow cytometry. CHOK1 and CHOR1.1 cells were plated out in a 24-well dish and transfected 24 h later with either TLR2 or TLR4 expression constructs using FuGENE6 transfection reagent. The constructs were obtained by cloning of the full-length TLR genes into pUNO vector, Invitrogen. Different ratios of transfection reagent to plasmid were used (3:1, 3:2, 6:1) to determine the best one for TLR expression. At least 72 h after transfection the CHOK1 and CHOR1.1 cells were harvested and stained with either FITC-TLR2 antibody, PE-TLR4 antibody, or FITC-PA. After staining the cells were fixed with 0.2% paraformaldehyde and examined using the flow cytometer.

CHOK1 cells did show significantly enhanced binding of FITC-PA compared to CHOR1.1 as it would be expected. However no differences were seen in either cell line when stained with FITC-PA after TLR2 or TLR4 transfection. No differences were seen in FITC-TLR2 or PE-TLR4 antibody staining of either the CHOK1 or the CHOR1.1 cell lines under any of the transfection conditions compared to unlabeled controls.

Detection of the PA ability to bind TLR2 and TLR4 by using immunoprecipitation assay.

In order to determine whether or not TLR-2 and TLR-4 receptor from cell extracts could bind PA protein, an experiment was performed in which TLR antibodies were used to pull whole receptor out of the extract, and then the receptor was exposed to PA protein to determine if it could be bound by the receptor.

Peripheral blood mononuclear cells (PBMCs) were isolated from a Red Cross donor via lymphocyte separation medium (Mediatech, Inc., Herndon, Va.), and were re-suspended in lysis buffer. The final protein concentration was adjusted to1 mg/ml. TLR-2 polyclonal antibody raised against clone TL2.1 (E-Biosciences) was covalently bound to an aminoalyl resin of the Seize Primary Kit (Pierce, Rockford, IL). TLR-4 polyclonal antibody raised against clone HTA125 (E-Biosciences) was also covalently bound to an identical aminoalyl resin. Each bound antibody and resin was incubated in PBMC extract for 30 min in a column, and the extract allowed to flow through, with the resin/Ab/bound protein complex remaining in the column. A solution of PA at 10 μg/ml in the same lysis buffer was then incubated for 30 min and allowed to flow through, followed by subsequent washes to elute nonspecifically-bound proteins. Bound components were then eluted and run on a 4-20% Tris-glycine polyacrylamide SDS-denaturing gel. Protein was transferred to a PVDF filter and incubated for 20 min at a 1:1000 dilution of murine monoclonal anti-PA antibody. Anti-PA antibody was detected with HRP-labeled goat anti-mouse antibody, and developed using ECL reagent (BIO-RAD, Hercules, CA).

No bands were observed on western blots performed with TLR-4 resin-bound antibody (data not shown). TLR-2 western blots showed a very strong band corresponding to between 82 and 90 kDa, and a band corresponding to between 52 and 55 kDa. An approximately 30 kDa band was also observed in the PA control lane, corresponding to a fragment of PA used for the study. The 82 kDa band is very strong and would correspond to the size of whole PA protein. The smaller band observed at 52 kDa likely corresponds to the association of the 30 kDa PA fragment with a 20 kDa, pre-activation cleavage protein from PA. (Fig. 3.2.6).

TLR-2's role as a semi-specific binder of various pathogen-associated molecules seems to fit with its potential to bind a PA protein preparation. Whether or not it actually recognizes and initiates an effector signal transduction is unknown, but would seem likely. Additionally, it is unknown whether or not PA's potential binding to TLR-2 can result in formation of the heptamer, and entry of LF into the cell, but it would appear that if indeed TLR-2 does bind PA, that an important goal of the bacterium would be to shut off the signal cascade started by TLR-2 activation, making TLR-2's potential as another proteinacious receptor for PA an intriguing possibility.

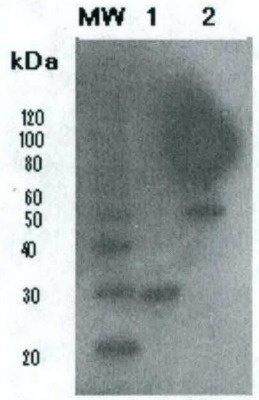


Fig. 3.2.6. Western Blot of immunoprecipitated (IP) TLR-2 bound to PA and detected by anti-PA antibody. Secondary antibody was HRP-labeled, and the blot was developed using ECL reagent. MW lane is a Western Standard MW marker, lane 1 is a PA control fragment, and lane 2 is the PBMC flow through from the TLR-2 IP/PBMC/PA mixture.

3.2.2. Possible Role of TLRs in the Infectious Process Caused by Vaccinia Virus in Cell Culture.

Effects of TLR signaling on the expression of the vaccinia late gene. We have adapted a vaccinia virus (VV) reporter assay, in which *E. coli lac*Z gene is inserted under the control of a late viral promoter. In this assay the amount of expressed *Lac*Z could be used viral quantification. In the initial experiments we found the optimal MOI (0.5 to 2) and the dilution of the lysate (1:100) for further studies (Fig. 3.2.7).

We then examined the effect of TLR signaling on the late viral gene expression. Our results indicated that transient expression of TLR-2, -3, -4, and -9 enhanced the late viral gene expression by 35% (Fig. 3.2.8). Both negative and positive effects of TLRs and their stimulants were detected. For example, induction of TLR-3 by PIC (polyinosine-polycytidine) increased viral gene expression by 28%. PGN induction in the presence of transiently expressed TLR-6 and TLR-2 led to 20% increase in the late viral gene expression compared to a slight decrease in the reporter gene expression in the presence of transiently expressed TLR-2. Further experiments are needed to optimize the conditions for the induction of other TLRs such as TLR4. In the same experiment, the effect of LTR signaling and vaccinia infection was also measured on NF-κB/ELAM-1 activation using SEAP (secreted embryonic alkaline phosphatase) assay following manufacture's instructions (InvivoGen, CA) (Table 3.2.1).

We found that TLR-3 induction by PIC had the most remarkable effect on the NF-κB/ELAM-1 activation, being 10-fold of that in the reporter-only-treated samples. VV infection provided additional six-fold activation.

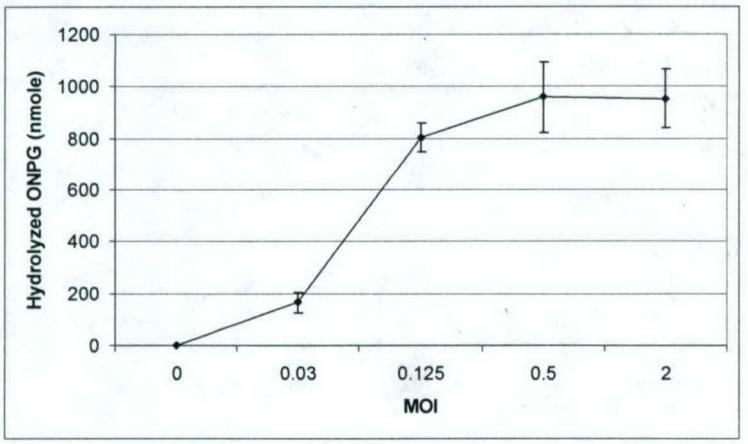


Fig. 3.2.7. Effect of MOI on β-galactosidase activity. HeLa cells were infected with a recombinant vaccinia virus expressing *lac*Z gene at various MOIs for 20 h. The cell lysates were assayed for β-galactosidase activity and expressed as means ± SDs for triplicate samples.

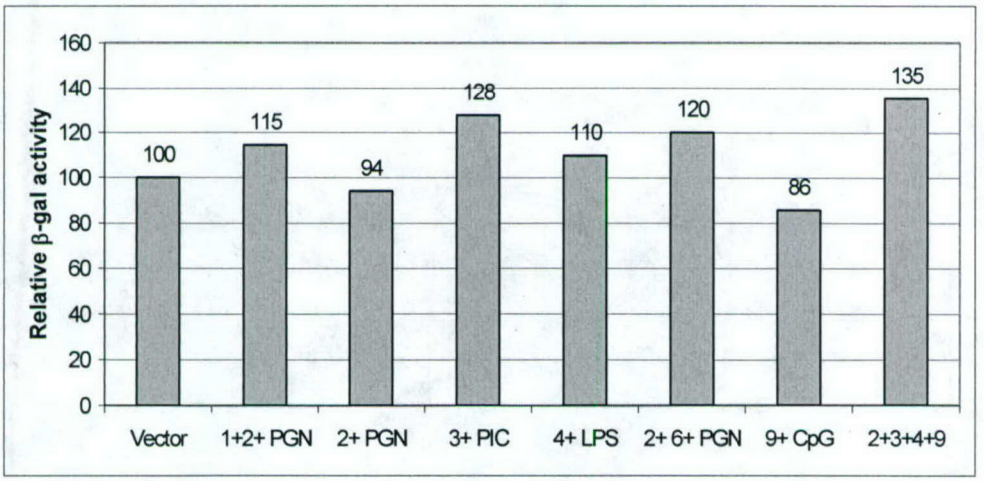


Fig. 3.2.8. Effect of TLR/TLR ligands on vaccinia late gene expression. HEK293T cells were transfected pUNO-hTLR plasmids (InvivoGen, CA) expressing indicated TLRs. Different combinations of the expression constructs were used for transfection. 20 h later the cells were infected with a recombinant VV expressing *E. coli* lacZ gene for 20 h in the presence or absence of the TLR ligand. The cell lysates were assayed for β-galactosidase activity and expressed as relative enzyme activity of infected and treated samples over the infected and untreated samples (no TLR expression). The X axis labels represent the TLR receptor number(s) and stimulants (PGN, peptidoglycan from *Bacillus alcalophilus*; LPS, lipopolysacharide from *E. coli*; LPS, lipoteichoic acid, CpG, deoxycytidyl 3'-5'-guanine, PIC, polyinosine-polycytidine complex.

Table 3.2.1. Effect of TLR ligand and VV infection on NF-κB activation (fold induction)

Ligand	TLR	-Virus	+Virus
PGN	2	2.1	2.2
PGN	2 + 1	2.0	2.9
PGN	2 + 6	2.4	4.1
PIC	3	10	16
LPS	4	1.05	1.34
CpG	9	0.8	1.2

Activation of TLR-2 and TLR-6 by PGN was higher in the presence of viral infection. It is clear from our initial observations that TLR stimilation may have a considerable influence on the VV multiplication. However, our induction conditions may not be optimal for certain TLRs. For more quantitative data, it is necessary to further optimize the conditions for each particular TLR/ligand system.

Effects of vaccinia infection and PGN on the expression of TLRs. To examine the effect of TLR-2 stimulation through peptidoglycan (PGN) on poxvirus infection, human monocyte THP-1 cells were treated with PGN and infected with VV. The THP-1 cells were stained with specific antibodies for flow cytometric analysis of the expression of TLR-2, TLR-3, and TLR-4. We found that VV infection and the PGN treatment downregulated the expression of TLR-2 on THP-1 cells (Fig. 3.2.9).

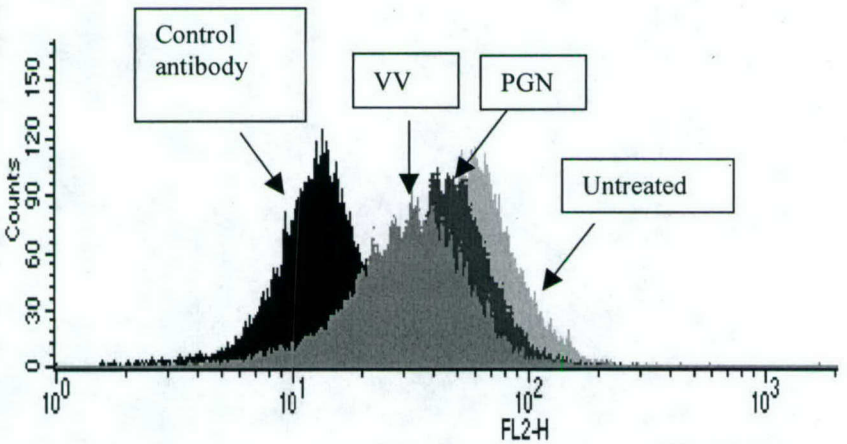


Fig. 3.2.9. Vaccinia infection and PGN stimulation down-regulate TLR2 expression on THP-1 cells. Human monocytic THP-1 cells were treated with *B. alcalophilus* PGN, infected with VV, or left untreated for 20 h. Staining was performed with PE-labeled anti-hTLR2 antibody or PE-isotype control antibody (eBiosciences, CA), and cells were analyzed by flow cytometry.

The expression levels of TLR-3 and TLR-4 (data not shown) were relatively low. And it is difficult to distinguish the difference in the levels of TLR-3 and TLR-4 between the control and treated samples.

TLR2 and PGN-induced IFN-α production of macrophages. We previously showed that *Bacillus alcalophilus* peptidoglycan (PGN) is capable of inducing IFN-α-mediated inhibition of vaccinia virus replication (Liu *et al.,* 2004). To examine the likely role of certain TLRs in this PGN-induced IFN-α production, we conducted antibody-blocking experiment. Our preliminary data showed that blocking of TLR2 enhanced IFN-α production; whereas pre-incubation of murine macrophage cells with anti-TLR4 or anti-TLR2 plus anti-TLR4 antibodies had no obvious effect on the PGN-induced IFN-α secretion (Fig. 3.2.10). Our data suggest that anti-TLR2 antibody may display stimulatory, but rather inhibitory, role in the PGN-induced IFN-α production. We plan to repeat this experiment with more doses of Abs. Unfortunately, these two anti-TLR antibodies are the only commercial ones for murine TLRs.

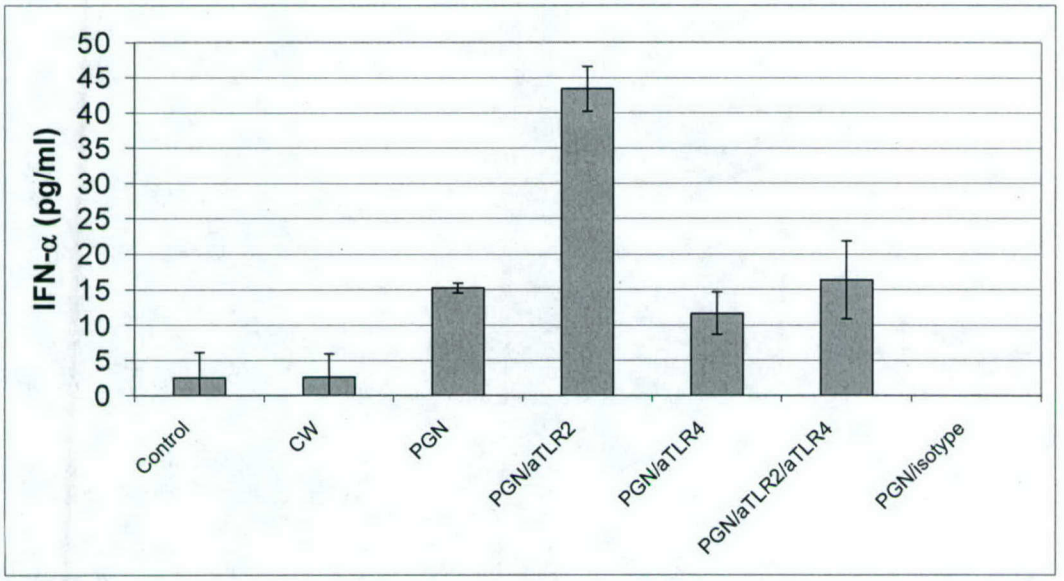
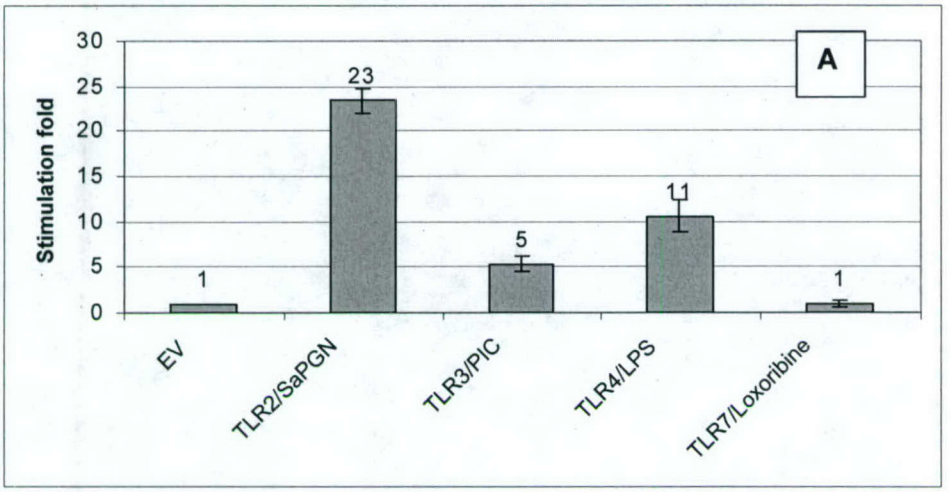


Fig. 3.2.10. Effect of anti-TLR antibodies on the PGN-induced IFN-α secretion. Murine macrophage RAW 264.7 cells were treated with anti-TLR2, anti-TLR4, anti-TLR2 + anti-TLR4, or isotype antibody for 1 h, then treated with *Bacillus alcalophilus* PGN for 20 h. The levels of murine IFN-α secreted to the supernatant were measure by ELISA and expressed as means ± SDs for triplicate samples.

Effects of TLR activation on NF-κB activation. To establish the relationship between TLR-mediated NF-κB activation and poxvirus infection, we adapted the luciferase assay to our lab conditions. This assay is more sensitive and more commonly used than the SEAP assay in TLR studies. Our data showed that the levels of NF-κB resulting from a 6-hour activation via TLR-2, -3, -

4 were higher than that from the 24-h activation (Fig. 3.2.11). Similar to our previous data with SEAP assays, no significant activation via TLR-7 and TLR-9 were observed.



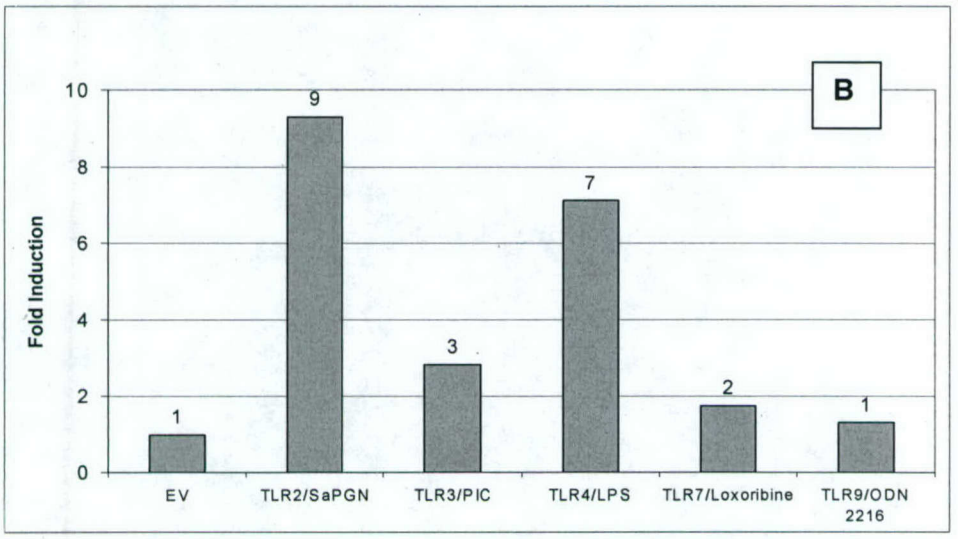


Fig. 3.2.11. Effect of TLR expression and signaling on the activation of NF-κB. HEK293 cells in triplicates were transfected with plasmids expressing indicated TLRs and luciferase reporter for 24 h, activated with the indicated TLR ligands for 6 h (5A) and 24 h (5B), and assayed for the luciferase activity. Values represent the relative stimulation fold.

Conclusions and suggestions. Experiments in Task 3 demonstrate that pretreatment of PBMCs with anti-TLR-4 antibodies inhibits PA-induced signaling, while anti-TLR2 antibody has an opposite effect. This data indicate a possibility of direct PA-TLR-4 interaction, although other possibilities cannot be currently excluded. Enhancement of cytokine release by anti-TLR-2 antibody needs to be confirmed further. We also plan to re-evaluate the antibody titers, as well their stimulatory and neutralizing activities.

Flow cytometry does not reveal a measurable amount of TLRs on cell surface and therefore this approach is not able to confirm the hypothesis on the direct interaction of TLRs and PA. Several possible reasons could result in the negative result for this experiment. First, it is obvious that the transfection efficiency was insufficient for reliable measurements. Further modification of the procedure or change of the vector is necessary. Second, the affinity between PA and TLRs could be rather low compared to the affinity between PA and other receptors such as ATR. We are planning to repeat the same experiment using different protocol.

Direct immunoprecipitation experiments provide evidence in favor of the PA interaction with TLR-2 but not TLR-4, however this result could be explained by low level of TLR-4 in resting human PBMCs. The experiments on TLR-4 expression in PBMCs in different conditions of stimulation would be useful for answering this question. Collectively, our data are consistent with the hypothesis that TLR may play important role in anthrax infection, and further studies are warranted to address several questions which arose from our experiments.

Our data regarding VV infectious process and TLRs indicate that certain TLR-mediated signaling pathways affect the expression of late viral genes. This may subsequently contribute to the outcome of poxvirus replication and pathogenesis. On the other hand, poxvirus infection and PGN inducers can down-regulate the expression of certain TLRs. In future studies we suggest to focus on the role of TLR-2, -4, and –3 in relation to poxvirus infections, as well as the in vivo role of TLR-2 and TLR-4 using transgenic and knockout animals using the TLR-deficient mice; and (3) to use siRNAs that target TLR-2 specific pathways to investigate its roles in NF-κB activation and poxvirus infection. Part of this data has been published in the journal FEMS Immunology and Medical Microbiology.

Materials and Methods

Cells and viruses. Murine macrophage cell line RAW 264.7 (ATCC # TIB-71) was maintained in Dulbecco's modified eagle medium (D-MEM/F-12) supplemented with 10% FetalClone I fetal bovine serum (FBS, Hycone), 100 units /ml of penicillin, and 100 μg/ml of streptomycin (GIBCO-BRL). African green monkey kidney BS-C-1 cells (ATCC # CCL-26) and human cervical adenocarcinoma HeLa cells (ATCC# CTL-2.2) were maintained in EMEM medium supplemented with 10% FBS, 100 units /ml of penicillin, and 100 μg/ml of streptomycin (GIBCO-BRL). VV (strain WR) was purchased from ATCC (VR-119), propagated in HeLa cells, and titrated on BS-C-1 cells.

Preparations of bacteria cell walls and PGN. Bacteria *Bacillus alcalophilus* Vedder (ATCC# 27647) was grown following ATCC's recommendations. Bacterial CW, PGN, and LTA were prepared following procedures adapted from those previously published (Papov et al., 2002). In brief, bacterial cultures were collected by centrifugation (25,000 X g for 20 min). The pellet (150 mg of wet weight) was then subjected to 3-4 cycles of boiling (4% SDS for 15-30 min) and centrifugation, resuspended in 30 ml of 2 M NaCl, centrifuged, washed with pyrogen-free distilled water, and centrifuged. The pellets were freeze dried, resuspended in pyrogen-free distilled water to a final concentration of 1 mg/ml, and stored at -80°C until used.

PGN was isolated from lyophilized CW following treatment with enzymes and hydrofluoric acid. Thirty milligrams of lyophilized CW were resuspended in 1 ml of 0.1 M Tris-HCl (pH 7.5), treated with α-Amylase (100 μg/ml) for 2 h, DNase and RNase (each at 100 μg/ml) for 2 h, and trypsin (100 μg/ml in 10 mM $CaCl_2$) for 16 h. After boiling for 15 min in 1% SDS, the samples were centrifuged at 40,000 X g for 15 min. The pellet was washed 3 times with distilled water, once with 8 M LiCl, centrifuged, washed four times with distilled water, and dried. Five milligrams of dry pellet were resuspended in 1 ml of hydroflouric acid (49% w/v), incubated for 48 h at 4°C, washed four times with distilled water, resuspended in 0.1 M Tris-HCl (pH 7.5), washed until pH was neutral. Following a centrifugation at 30,000 X g for 30 min, the pellet was treated with alkaline phosphatase (250 μg/ml) in 0.1 M $(NH_4)_2CO_3$ for 16 h at 37°C. After boiling for 5 min and washing twice with sterile distilled water, PGN was resuspended in sterile distilled water and stored at -20°C.

An important practical consideration in preparing and testing PGN or LTA is to avoid contamination by LPS from Gram-negative bacteria, which share most of the biological activities of CW components. Therefore, the absence of Gram-negative endotoxin in all the above preparations were confirmed using QCL-1000® Limulus Amebocyte Lysate (BioWhittaker) (Young et al., 1972). The DNA and protein contaminations in the above preparations were less than 1% and 3%, respectively.

Luciferase assay. HEK293 cells (2 x 10^4 cells per well) were seeded in a 96-well plate and were transfected on the following day with pUNO-MCS, TLR-2 (100 ng), TLR-3 (100 ng), TLR-4 (100 ng), in combination with luciferase reporter plasmid NF-κβ firefly (CloneTech, CA) (80 ng/well). Renilla luciferase reporter plasmid (Promega) (5 ng/well) was used as an internal control. Also added was 4% Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as a transfection enhancer. After a 24 hr. incubation, ligands were added to activate their respective TLRs (SaPGN (10 μg/ml), PIC (50 μg/ml), and LPS (1 μg/ml)). Plate was incubated again for 24 hr. and Dual-Glo Luciferase Assay (Promega, Madison, WI) was performed according to manufacture's instructions.

IFN-α ELISA. The levels of murine IFN-α secreted to the cell culture supernatant were measured in triplicate by an antibody-sandwich IFN-α ELISA kit (PBL Biomedical Laboratories) and a BD OptEIA™ ELISA set (BD PharMingen), respectively, following the manufacturer's instructions. The detection limit for IFN-α was 12.5 pg/ml.

3.2.3. Generation of Soluble TLRs as Potential Therapeutics

Soluble recombinant TLR proteins represent attractive tools to investigate the involvement of TLRs in the infectious process. However, the experimental approaches need to be developed to produce the proteins in quantities sufficient for testing. We have attempted bacterial recombinant expression of two TLRs, namely TLR-2 and TLR-4. The results obtained are similar for both of these receptors. Below we carefully describe the expression data.

.***Results.*** Oligonucleotide primers were chosen to amplify the extracellular domain of the Toll-Like Receptor 2 (TLR-2) gene starting from just outside the transmembrane domain which was predicted by the Simple Modular Architecture Research Tool or S.M.A.R.T (Fig. 3.2.12).

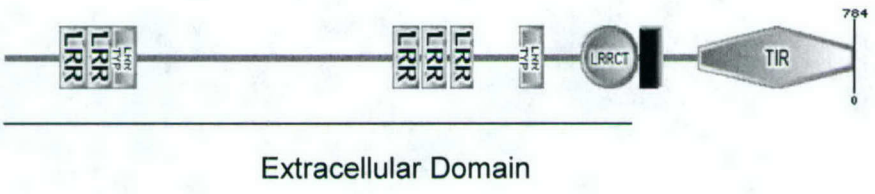


Fig. 3.2.12. Human TLR-2 domain structure.

Due to the absence of introns in this specific gene, this region was amplified directly from human THP-1 cells (ATCC, #TIB-202). PCR of this domain is shown in Fig. 3.2.13.

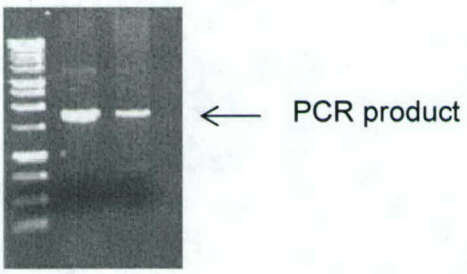


Fig. 3.2.13. The TLR-2 PCR amplification product.

The PCR product was cloned into pTrcHis2 TOPO® TA expression vector (Invitrogen, CA) according to the manufacturer's recommendations. This kit makes use of a myc epitope for detection with anti-myc antibody and a six-histidine residue for purification on an affinity column.

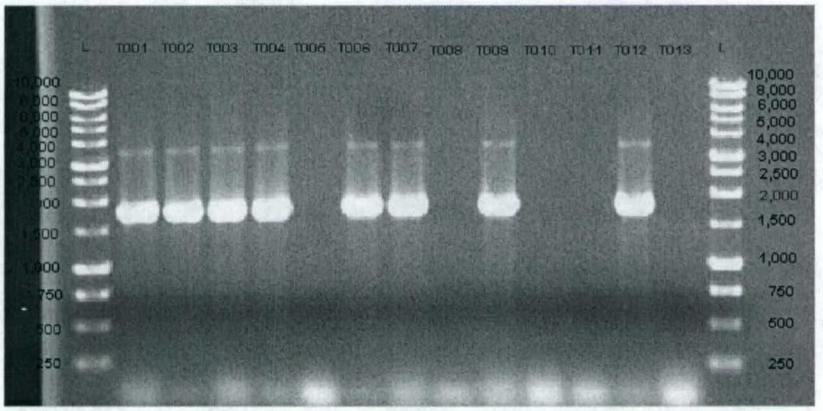


Fig. 3.2.14. Identification of TLR-2-positive clones using PCR with specific primers.

Thirteen colonies were selected for analysis via PCR with TLR2 primers to check for the insertion of the product into the pTrcHis2 vector. Eight clones showed to be positive for the insertion (Fig. 3.2.14). After conformation of positive clones, analysis was preformed to check for orientation of the insert. Analysis was done by first constructing plasmid maps (using Clone Map™) to analyze restriction sites to reveal the orientation of the insert. It was determined that an insert in the positive (forward) orientation, cut with EcoRI would yield 150 and 5992 base pair fragments on the gel. In the reverse orientation, 4517 and 1625 base pair fragments would result. Results of this analysis can be seen in Fig. 3.2.15. All orientation-positive clones were grown.

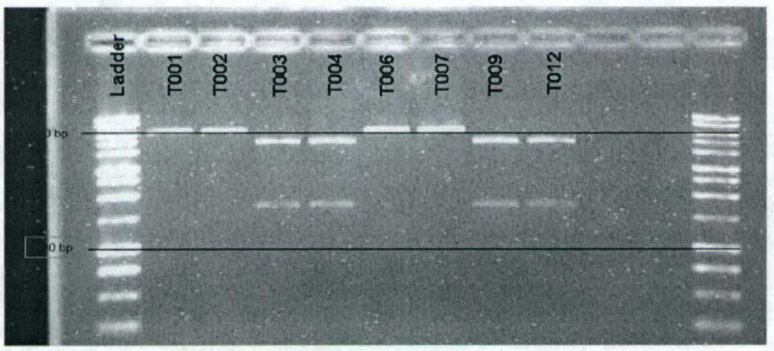
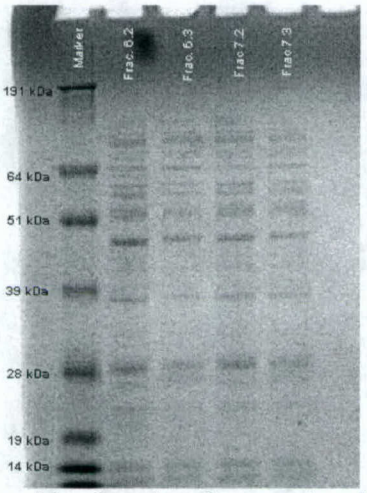


Fig. 3.2.15. Analysis of insert orientation in TLR-2-positive clones

Two of the positive insert clones were selected (T006 and T007 above) to be grown up and expressed in order to harvest protein. The culture was grown to an O.D. of 0.8 and induced with a final concentration of 1.0mM IPTG. Time points were taken before induction and then again every

hour for 5 hours to disclose optimal time for harvesting the protein of interest (Fig. 3.2.16). It was decided to try an initial purification after the full 5 hours to determine if the protein could be purified.

Fig. 3.2.16. Expression of recombinant TLR-2 in E.coli cells. Arrows indicate the expected size of the product.



The cell lysate was prepared by thawing the previously induced cells, resuspending and subjecting to both lysozyme and sonication treatments as means of lysis. The lysate was spun down, supernatant collected and stored at 4°C. The pellet was saved at -20°C for further analysis of possibly insoluble protein. Purification proceeded by means of an affinity column with nickel-based resin (Invitrogen®, ProBond™). The supernatant was loaded through the column, washed, and eluted with high imidazole concentration and the fractions were analyzed via SDS-PAGE analysis (Fig. 3.2.17).

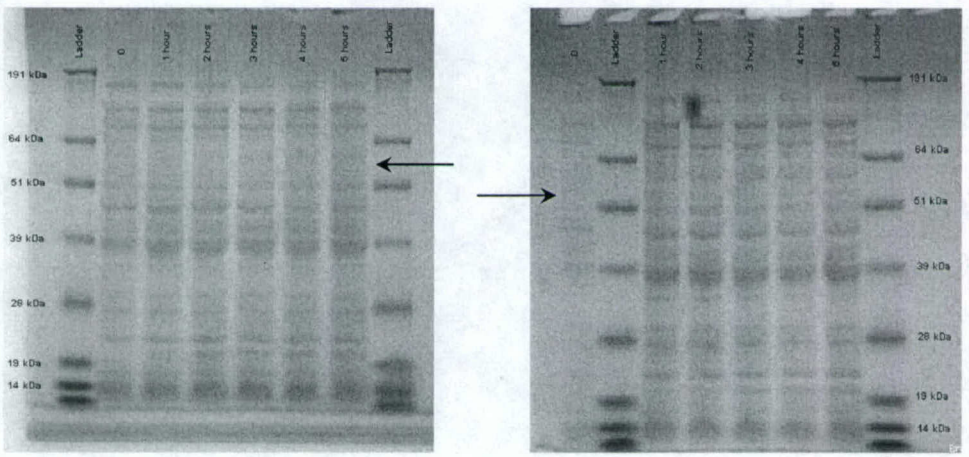


Fig. 3.2.17. SDS-PAGE of the column eluate after TLR-2 experession.

As can be seen from the picture, the fractions were much more contaminated than had been expected. Debris from the initial cell lysate preparation was run to check for presence of the protein

in the insoluble fraction; no significant amount was found. The plasmids were isolated from both T006 and T007 clones and sent for sequencing by Retrogen, Inc to confirm that no significant mutations that might result in a mis-translated product had occurred, of which none were detected. Following the sequencing results, it was determined that experiments needed to be preformed to further detect expression of our protein to be certain we were expressing enough to warrant further attempts at purification. Initial tests proceeded by thawing out more of the aforementioned 5 hour induction, boiling, and running the total cell extract through an SDS-PAGE gel to detect recombinant protein, using an in-gel His Tag stain (Invitrogen). There was a significant amount of truncated products found among the expression products. It was determined from the results of the previous gels that the induction time should be decreased, as truncated products which were showing up on the gels could be a result of degradation inside the host. Expression time was decreased to 3 hours and the same experiments were repeated.

Seeing that cleavage was still occurring in the host, it was decided to isolate our cloned construct and transfer it to *lon*$^-$/*omp*$^-$, BL-21 cells (Stratagene) which are deficient in both the Lon and Omp proteases implicated in degradation of some recombinant proteins. Transformation was

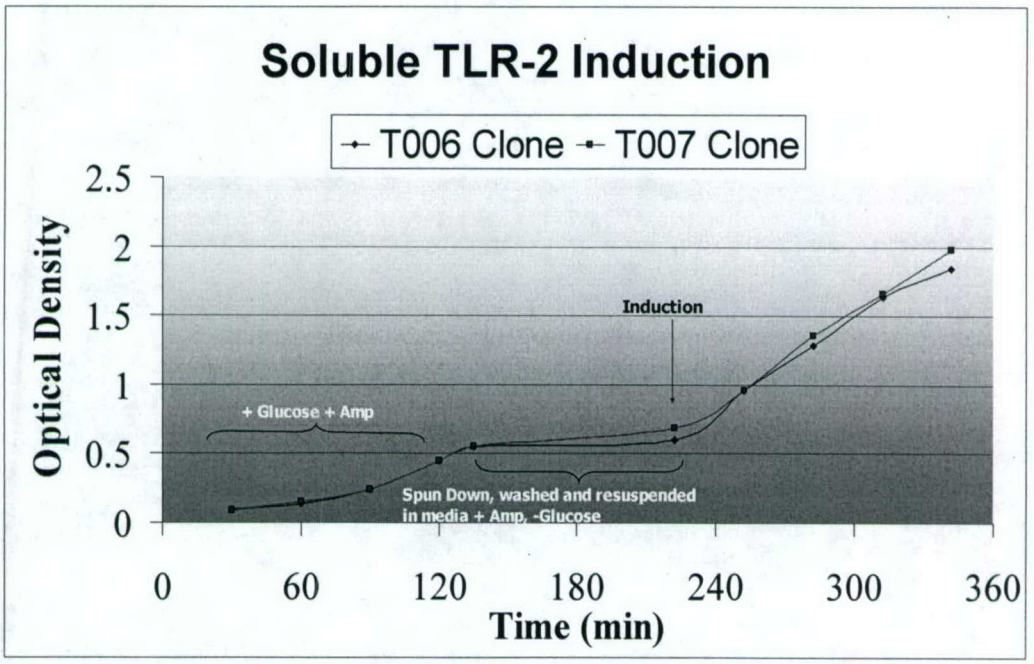


Fig.3.2.18. Growth curves for TLR-2 recombinant clones

successful and these new cells were grown up and induced in a manner similar to what was done for the competent E. coli described previously. In addition, it needed to be taken into consideration that our protein could be toxic to the host, which could explain the low signal on Westerns and why it was being degraded. This hypothesis was tested by inoculating media supplemented with 0.5% glucose to repress any basal level transcription. After the culture had reached an O.D. of 0.5, it was

spun down, glucose media was taken off and cells were resuspended in media without glucose and induced for the expression of TLR2. Time points were taken every 30 minutes for 5 hours to determine the toxicity of the expressed protein. The graph of growth based on optical density shows an exponential curve in growth expected in normal culture conditions (Fig. 3.2.18). Though the culture seemed to have been growing normally, the gels still showed a picture of cleavage and impure expression even in the BL-21 host. In an attempt to elute/purify the full soluble protein with the size closer to 70 kilodaltons and clean up some of the product, the elution was carried out over 25 fractions of increasing imidazole concentration (Fig. 3.2.19, left). The results were similar to what was seen before with the 4-fraction elutions, with several bands appearing upon purification and major bands showing up below the expected size of our protein. Though it was assumed that these were bands resulting from the cleavage of the expressed TLR2, it was confirmed by western blot (Fig. 3.2.19, right). The next logical step was to adjust the culture conditions of the expressing culture to further optimize the yield of the full soluble portion of the TLR and decrease the cleavage. The next set of experiments attempted this by adjusting the temperature of the culture during expression. The idea, which has been well described in the literature, is that as the temperature decreases, proteolytic activity will decrease as well. It was decided to lower the growth temperature to 28°C from 37°C. The cleavage was still apparent in the 28°C culture. These experiments showed a decreased amount of cleavage, but an extremely small amount of gain in overall production of full soluble length protein.

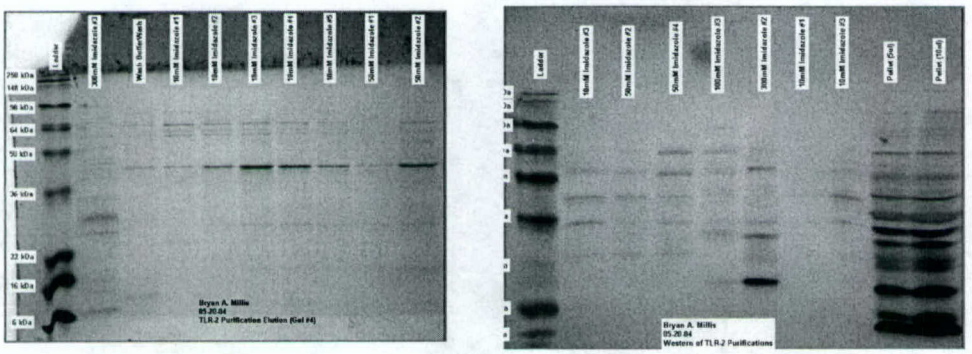


Fig.3.2.19. SDS PAGE (left) and Western blot with anti-myc antibodies of the same gel (right).

In addition to changing the temperature conditions for the growing cultures, different media formulations were used to analyze possible increases in protein production. A total of six different media formulations were tested for an increase in recombinant protein production (LB, LB + Leucine, Superior Broth, Super Broth, Power Broth, and Turbo Broth) with Turbo and Power coming out with the best return in total recombinant protein production (All media were from US Biological)

We also tested a hypothesis that the observed results were caused by protein translation being halted prematurely for some reason. In addition, we also suggested that codon bias problem might has significantly influenced the expression. The principle is that any organism has an abundance of tRNAs that supply commonly appearing triplet codons with amino acids during translation. If the organism is made to express a gene containing triplet codons which do not normally occur within the natural genome of the host, several adverse consequences can be observed. Of these include translational stalling, halting, frameshifting, and misincorporation. Bioinformatic analysis was performed with our protein compared with triplet codons known to be in rare supply within E. coli. Graphs were put together to show the breakdown of amino acids that are incorporated in our desired protein as well as the percentage of each of these amino acids that are encoded by five traditionally rare codons within E. coli. When the codon distribution was considered for the first 4 (of 5) amino acids, there seemed to be a very small percentage encoded by rare codons. When the 5th amino acid was analyzed (arginine) it was found that out of all the arginines in our desired protein 85.71% were encoded by rare tRNAs (Fig. 3.2.20).

In order to alleviate this codon bias problem an E. coli strain from Novagen, Rosetta 2, was used which has an incorporated plasmid encoding 7 rare tRNAs for E. coli. These cells were transformed with the TLR2 construct and expressed under 3 different temperature conditions (28°C, 32°C, and 37°C shown below respectively) and in 2 different types of media, LB and Power Broth (Fig. 3.2.21).

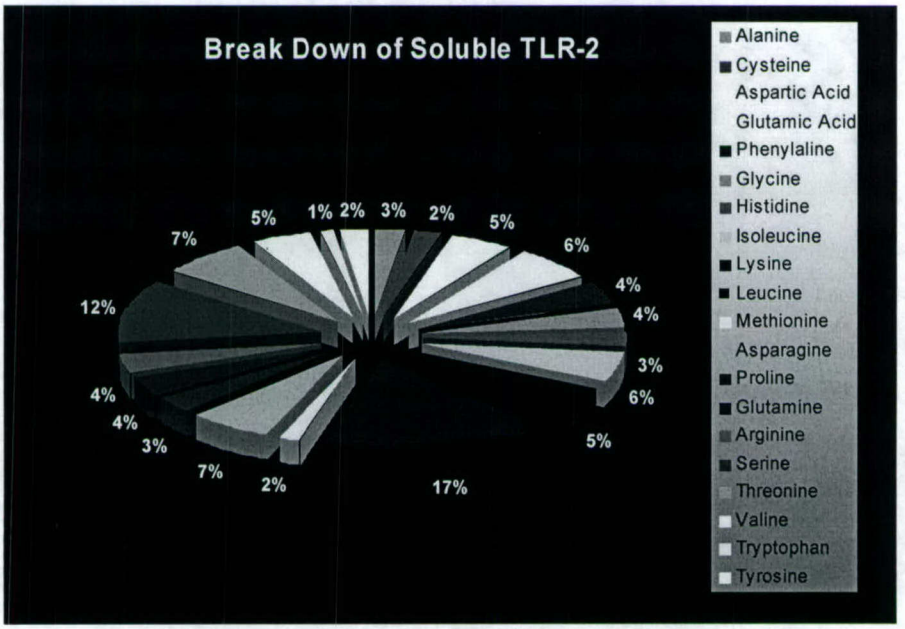


Fig. 3.2.20. Codon distribution in the human TLR-2 gene.

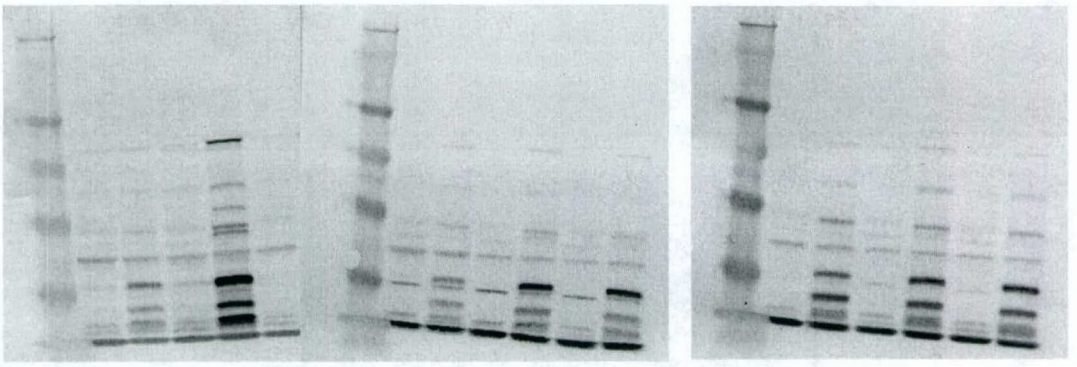


Fig. 3.2.21. SDS-PAGE of recombinant TLR-2 expression in different conditions (see text).

Though the significance of temperature decrease on proteolytic activity can be observed nicely above, the appearance of the cleavage products still was shown to be a problem. A decision was made to try to clone the same protein into an N-terminal vector to see if somehow switching the tag may have an effect on the stability of the protein. The sTLR2 was cloned into pTrcHis TOPO TA expression vector (Invitrogen). In addition to the full soluble domain of TLR2, fragments were also cloned from the receptor to try to isolate smaller portions of the gene, which still might retain binding and functional capabilities useful for our experiments (Fig. 3.2.22). No degradation is seen

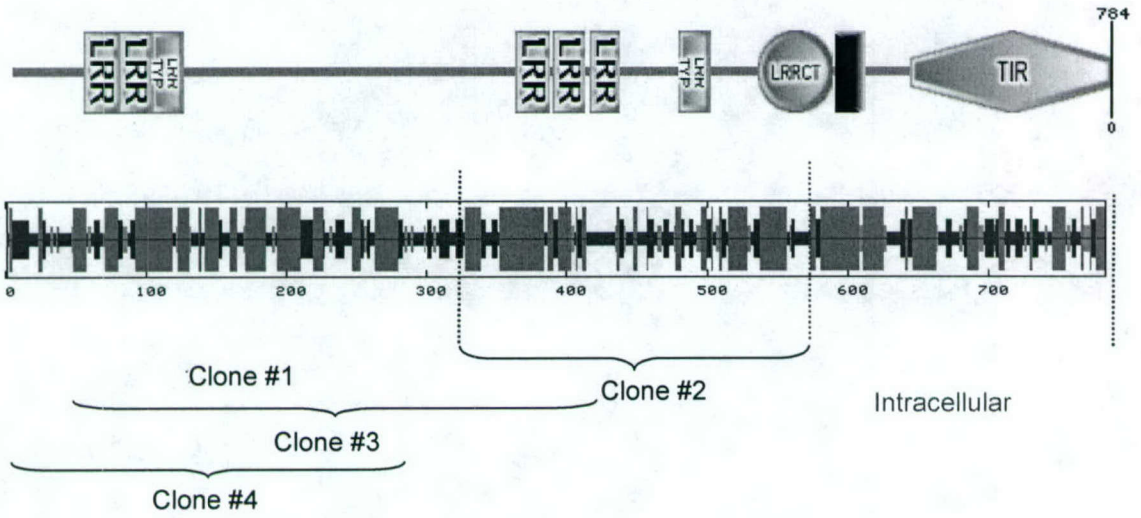


Fig. 3.2.22. Schematics of the TLR-2 gene subclones in the N-terminal tag vector.

in the blot of expressed cells (Fig. 3.2.23). However, the purification of proteins from cells revealed a complex picture (Fig.3.2.24). As can be seen the ladder of purified products is still very apparent. These results have not yet found a plausible explanation.

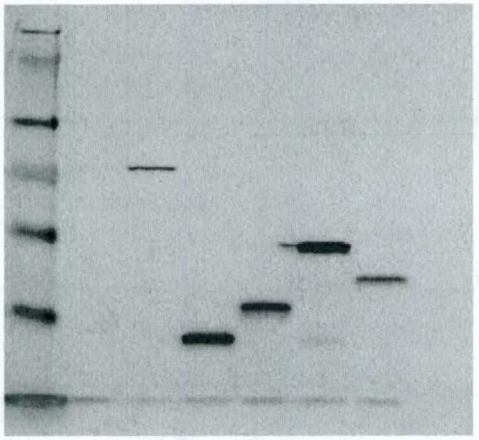


Fig. 3.2.23. Western blot of N-terminal tag proteins. Lanes represent:1, MW ladder; 2, uninduced sTLR2; 3, induced sTLR2; 4, clone #1; 5, clone #2; 6, clone #3; 7, clone #4.

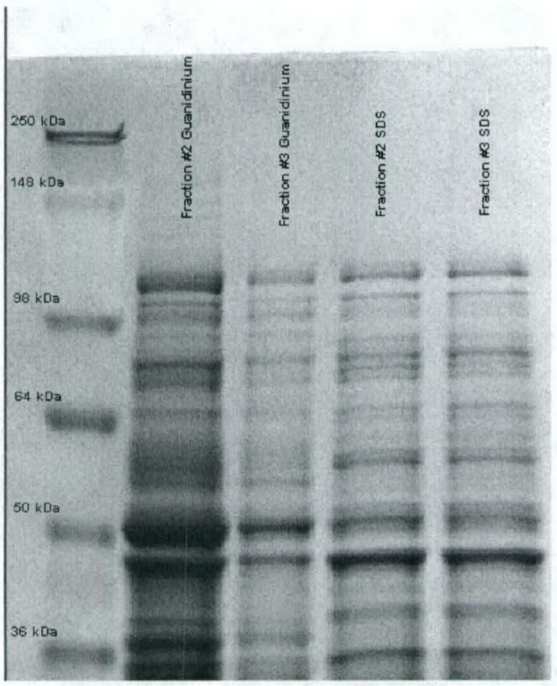


Fig.3.2.24.The fractions of the TLR recombinant proteins expressed with N-terminal tag and purified under denaturing conditions with guanidinium HCL and SDS. lanes on a gel represent: 1, MW marker; 2 and 3, fractions eluted from Ni-column in presense of guanidinium HCl; 4 and 5, fraction eluted in presense of SDS.

Conclusions and suggestions. Current theories are investigating the presence of an as of yet unknown ligand for TLR2 within E. coli. This ligand may be binding to the soluble receptor upon cell lysis and purification, and co-eluting with it as well. Upon running the samples on a denaturing gel the receptor-ligand complex can dissociate, resulting in the ladder of purified products. Current research trends include further purification techniques as well as sequencing of the purified

products to attempt to identify previously unknown E. coli ligands for TLR2. In addition, as reported in the literature, several small domains within the extracellular portion of TLR2 have been characterized as being functional in binding with traditional ligands for the receptor. Synthetic peptide generation in combination with transfection experiments with TLR2 are underway to determine if these peptides could compete with both ligands already described in the literature, as well as microbial pathogens implicated in the TLR2 pathway.

KEY RESEARCH ACCOMPLISHMENTS

Task 1

- Two classes of compounds capable of increasing the protective efficacy of anthrax antibiotic treatment have been discovered, namely the apoptosis inhibitors and the adenosine receptor agonists. These compounds have been previously tested in pre-clinical studies, and can serve as drug leads for further pharmaceutical development on new anti-anthrax preparations.
- Novel compounds for inhibition of lethal toxin activity, namely aminocyclodextran derivatives, have been suggested, synthesized and tested in cell culture. Accute toxicity and protective efficacy of these compounds in combination with ciprofloxacin has been evaluated.

Task 2

- Bioinformatic analysis of anthrax genome has been carried out in order to identify potential virulence-enhancing factors, in addition to known anthrax toxins.
- Anthrax secreted proteolytic enzymes have been evaluated in vivo and in animal challenge experiments as virulence-enhancing factors.
- Acute toxicity and hemorrhagic activity of anthrax proteolytic proteins in mice have been demonstrated.
- Anthrax treatment approach based on inhibition of proteolytic activity using chemical inhibitors has been suggested and experimentally evaluated in murine model. High efficacy of combination antibiotic-protease inhibitor treatment has been demonstrated.
- Rabbit immune sera have been raised against selected epitopes of anthrax proteolytic enzymes and its neutralizing activity has been demonstrated.
- Anthrax treatment approach based on inhibition of proteolytic activity using hyperimmune serum has been suggested and experimentally evaluated in murine model. High efficacy of combination antibiotic-anti-protease serum treatment has been demonstrated.
- Antibiotic-protease inhibitor and antibiotic-anti-protease antibody formulations have been suggested for further development of new pharmaceuticals which may include already approved or pre-clinically-studied drugs with anti-proteolytic and antibiotic activities.
- Conclusion on anthrax collagenases and elastases, belonging to M4 and M9 families, as anthrax virulence factors has been confirmed in animal protection experiments.

Task 3

- Evidence on the involvement of TLR in lethal toxin activity has been obtained using anti-TLR antibodies and cells in culture.

- Evidence on the direct interaction of anthrax PA with TLR-2 has been obtained using immunoprecipitation technique.
- Enhancement of cytokine release induced by PA in cultures cells in the presence of anti-TLR-2 antibody has been observed.
- Genetic constructs have been designed for expression of soluble, truncated, as well as full-length TLR-2 and TLR-4 in eukaryotic and prokaryotic systems. Isolation of soluble receptors expressed in E.coli cell has been attempted.
- Effect of TLR-mediated pathways on interferon-α gene induction causing the inhibition of vaccinia virus replication in cultured cells has been discovered.
- Effect of vaccinia virus infection on downregulation of certain TLRs expression has been demonstrated.
- It has been concluded that TLR signaling can potentially contribute to the outcome of poxvirus replication and pathogenesis.

REPORTABLE OUTCOMES

1. One article has been published: Liu G, Zhai Q, Schaffner D, Popova T, Hayford A, Bailey C, Alibek K. (2004) Bacillus alcalophilus peptidoglycan induces IFN-alpha-mediated inhibition of vaccinia virus replication. *FEMS Immunol Med Microbiol* 2004; 42(2):197-204.
2. One article has been submitted for publication in the Antimicrobial Agents and Chemotherapy journal, September, 2004.
2. Three abstracts have been presented at the American Microbiological Society Biodefense conference in 2004.
3. Two abstracts have been submitted for the upcoming NCBC - GMU -The Burnham Institute conference, Crossing Boundaries, Washington DC, November, 2004.
4. Five research proposals have been submitted to the NIH in response to biodefense solicitations.

CONCLUSIONS

Research under current project has been carried out in three interconnected directions aimed to develop new therapeutic approaches against biological weapon threat agents, namely Bacillus anthracis and smallpox virus (using vaccinia virus model).

We have successfully demonstrated new previously unexplored directions to improve anthrax antibiotic therapy by using a combination of antibiotic with apoptosis inhibitors and adenosine receptor agonists.

New candidate virulence factors of B. abthracis have been suggested based on our bioinformatics analyses. Experiments on anti-protease therapy in murine model confirmed this

suggestion and demonstrated high efficacy of treatments with protease inhibitors, as well as anti-protease antibodies. This direction of research seems very promising in both practical and theoretical aspects. Detailed investigation of the pathogenic proteases of B. anthracis needs to be continued in order to get further insight into the mechanisms of disease.

Evidence on toll-like receptor participation in both infections caused by B. anthracis and vaccinia virus has been obtained. This particular area of microbiological research is expected to result in important discoveries regarding the host innate immune response. We suggest further research in this direction in order to substantiate and further develop our findings.

Recombinant soluble proteins based on the toll-like receptors seem to be promising model agents. Difficulties associated with their production in bacterial recombinant system slowed our progress in this direction. To circumvent these problems we suggest to reformulate this approach. It seems that expression of TLR functional domains, rather than a whole protein molecule, as well as direct chemical synthesis of mimic peptides could be considered as future alternatives.

References

Abramova FA, Grinberg LM, Yampolskaya OV and Walker DH (1993). Pathology of inhalational anthrax in 42 cases from the Sverdlovsk outbreak of 1979. Proc Natl Acad Sci U S A. 90(6):2291-4.

Akesson A., Hedstrom SA, and Ripa T (1991). Bacillus cereus: a significant pathogen in postoperative and post-traumatic wounds on orthopaedic wards. Scand. J. Infect. Dis. 23(1):71-7.

Akira S, Takeda K, Kaisho T. (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* 2001; 2:8 675-680.

Barrett AJ (2004) Bioinformatics of proteases in the MEROPS database. 2004. Curr Opin Drug Discov Devel 7(3):334-41.

Biagini A., and Puigserver A. (2001). Sequence analysis of the aminoacylase-1 family. A new proposed signature for metalloexopeptidases. Comp Biochem Physiol B Biochem Mol Biol 128(3):469-81.

Blander JM, Medzhitov R (2004) Regulation of phagosome maturation by signals from toll-like receptors. Science.304(5673):1014-8.

Bonventre PF, and Eckert NJ (1963). The biologic activities of Bacillus anthracis and Bacillus cereus culture filtrates. 1963. Am J Pathol;43:201-11.

Brown SB, Savill J (1999) Phagocytosis triggers macrophage release of Fas ligand and induces apoptosis of bystander leukocytes. *J Immunol* 1999; 162:1 480-485.

Chiarugi A, Calvani M, Meli E, Traggiai E, Moroni F (2001) Synthesis and release of neurotoxic kynurenine metabolites by human monocyte-derived macrophages. *J Neuroimmunol* 2001; 120(1-2):190-198.

Cui X, Moayeri M, Li Y, Li X, Haley M, Fitz Y, Correa-Araujo R, Banks SM, Leppla SH, and Eichacker PQ (2004) Lethality during continuous anthrax lethal toxin infusion is associated with circulatory shock but not inflammatory cytokine or nitric oxide release in rats. *Am J Physiol Regul Integr Comp Physiol* 286(4):R699-709.

Cleary PP, Prahbu U, Dale JB, Wexler DE, and Handley J (1992) Streptococcal C5a peptidase is a highly specific endopeptidase. 1992. *Infect Immun* 60(12):5219-23.

David DB, Kirkby GR, and Noble BA (1994) Bacillus cereus endophthalmitis. *Br J Ophthalmol* 78(7):577-80.

Drobniewski FA (1993) Bacillus cereus and related species. Clin Microbiol Rev 6(4):324-38.

Earl, P.L., Cooper, N., Wyatt, L.S., and Moss, B. (1998) Titration of vaccinia virus stocks by plaque assay. 16.16.5-16.16.6. In: F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, (Eds.), *Current Protocols in Molecular Biology*, Vol. 3, pp. 16.16.5-16.16.6. John Wiley & Sons, Inc., USA.

Eckert NJ, and Bonventre PF (1963) In vivo effects of Bacillus anthracis culture filtrates. *J Inf Dis* 112:226-32.

Erwin JL, DaSilva LM, Bavari S, Little SF, Freidlander AM, Chanh TC. (2001) Macrophage-Derived Cell Lines Do Not Express Proinflammatory Cytokines after Exposure to *Bacillus anthracis* Lethal Toxin. *Infect Immun* 2001; 69:2 1175-1177.

Ezepchuk Iu.V., E.D. Bobkova, and N.K. Prosvetova 1969. [On the toxic and various biochemical properties of filtrates of *B. anthracis* and cereus] *Zh. Mikrobiol. Epidemiol. Immunobiol.* 46(6):19-23 [Article in Russian]

Fishman P, Bar-Yehuda S, Farbstein T, Barer F, Ohana G.J (2000) Adenosine acts as a chemoprotective agent by stimulating G-CSF production: a role for A1 and A3 adenosine receptors. *Cell Physiol.* 183(3):393-8.

Friedlander AM (2001) Tackling anthrax. *Nature* 414(6860):160-1.

Fish DC, Klein F, Lincoln RE, Walker JS, Dobbs JP. (1968) Pathophysiological changes in the rat associated with anthrax toxin. *J Infect Dis* 1968; 118:1 114-124.

Goldenberg I, Shainberg A, Jacobson KA, Shneyvays V, Grossman E (2003) Adenosine protects against angiotensin II-induced apoptosis in rat cardiocyte cultures. *Mol Cell Biochem.* 252(1-2):133-9.

Grinberg LM, Abramova FA, Yampolskaya OV, Walker DH, Smith JH. (2001) Quantitative pathology of inhalational anthrax I: quantitative microscopic findings. *Mod Pathol* 2001; 14:5 482-495.

Guidi-Rontani C, Weber-Levy M, Labruyere E, Mock M. (1999) Germination of *Bacillus anthracis* spores within alveolar macrophages *Mol Microbiol* 1999; 31 9-17.

Hanna PC, Acosta D, Collier RJ. (1993) On the role of macrophages in anthrax. *Proc Natl Acad Sci USA* 1993; 90:21 10198-10201.

Hanna PC, Kruskal B, Ezekowitz R, et al. (1994) Role of macrophages oxidative burst in the action of anthrax lethal toxin. *Mol Med* 1994; 1 7-18.

Helgason E, Okstad OA, Caugant DA, Johansen HA, Fouet A, Mock M, Hegna I, and Kolsto J (2000) Bacillus anthracis, Bacillus cereus, and Bacillus thuringiensis--one species on the basis of genetic evidence. *Appl. Environ. Microbiol.* 66(6):2627-30.

Hoffmaster AR, Ravel J, Rasko DA, Chapman GD, Chute MD, Marston CK, De BK, et al. (2004) Identification of anthrax toxin genes in a Bacillus cereus associated with an illness resembling inhalation anthrax. *Proc. Natl. Acad. Sci. U S A.* 101(22):8449-54.

Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Friedlander AM, Hauer J, McDade J, Osterholm MT, O' Toole T, Parker G, Perl TM, Russell PK, Tonat K. (1999) Anthrax as a biological weapon: medical and public health management. Working Group on Civilian Biodefense. *JAMA* 1999; 281 1735-1745.

Ivanova N., A. Sorokin, I. Anderson, N. Galleron, B. Candelon, V. Kapatral, A. Bhattacharyya, G. Reznik, N. Mikhailova, A. Lapidus, L. Chu, M. Mazur, E. Goltsman, N. Larsen, M. D'Souza, T. Walunas, Y. Grechkin, G. Pusch, R. Haselkorn, M. Fonstein, S.D. Ehrlich, R. Overbeek, and N. Kyrpides (2003) Genome sequence of Bacillus cereus and comparative analysis with Bacillus anthracis. *Nature* 423(6935):87-91.

Kaminishi H, Hamatake H, Cho T, Tamaki T, Suenaga N, Fujii T, Hagihara Y, and Maeda H (1994) Activation of blood clotting factors by microbial proteinases. *FEMS Microbiol. Lett.* 121(3):327-32.

Kiener PA, Davis PM, Rankin BM, Klebanoff SJ, Ledbetter JA, Starling GC, Liles WC. (1997) Human monocytic cells contain high levels of intracellular Fas ligand: rapid release following cellular activation. *J Immunol* 1997; 159:4 1594-1598.

Kim J, Ochoa MT, Krutzik SR, Takeuchi O, Uematsu S, Legaspi AJ, Brightbill HD, Holland D, Cunliffe WJ, Akira S, Sieling PA, Godowski PJ, Modlin RL (2002) Activation of toll-like receptor 2 in acne triggers inflammatory cytokine responses. *J Immunol* 169(3):1535-41

Klein F., J.S. Walker, D.F. Fitzpatrick, R.E. Lincoln, B.G Mahlandt., W.I. Jones Jr, J.P. Dobbs, and K.J. Hendrix (1966). Pathophysiology of anthrax. *J Infect Dis* 116(2):123-38.

Klichko V.I., J. Miller, A. Wu, S.G. Popov, and K. Alibek (2003) Anaerobic induction of Bacillus anthracis hemolytic activity. *Biochem Biophys Res Commun* 303(3):855-62.

Komiyama T., H. Suda, T. Aoyagi, T. Takeuchi, and H. Umezawa (1975) Studies on inhibitory effect of phosphoramidon and its analogs on thermolysin. *Arch Biochem Biophys* 171(2):727-31.

Kon Y, Tsukada H, Hasegawa T, Igarashi K, Wada K, Suzuki E, Arakawa M, and Gejyo F (1999). The role of Pseudomonas aeruginosa elastase as a potent inflammatory factor in a rat air pouch inflammation model. FEMS Immunol Med Microbiol 25(3):313-21.

Kunst F, Ogasawara N, Moszer I, Albertini AM, Alloni G, Azevedo V, Bertero MG et al. (1997) The complete genome sequence of the gram-positive bacterium Bacillus subtilis. *Nature* 390(6657):249-56.

Lovgren A, Zhang M, Engstrom A, Dalhammar G, and Landen R (1990) Molecular characterization of immune inhibitor A, a secreted virulence protease from Bacillus thuringiensis. *Mol Microbiol* 4(12):2137-46.

Liu G, Zhai Q, Schaffner D, Popova T, Hayford A, Bailey C, Alibek K (2004) Bacillus alcalophilus peptidoglycan induces IFN-alpha-mediated inhibition of vaccinia virus replication. *FEMS Immunol Med Microbiol* 2004;42(2):197-204.

Maeda H, Akaike T, Sakata Y, and Maruo K (1993) Role of bradykinin in microbial infection: enhancement of septicemia by microbial proteases and kinin. *Agents Actions Suppl* 42:159-65.

Matute-Bello G, Liles WC, Steinberg KP, Kiener PA, Mongovin S, Chi EY, Jonas M, Martin TR (1999) Soluble Fas ligand induces epithelial cell apoptosis in humans with acute lung injury (ARDS). *J Immunol* 1999; 163:4 2217-2225.

Matute-Bello G, Liles WC, Frevert CW, Nakamura M, Ballman K, Vathanaprida C, Kiener PA, Martin TR (2001) Recombinant human Fas ligand induces alveolar epithelial cell apoptosis and lung injury in rabbits. *Am J Physiol Lung Cell Mol Physiol* 2001; 281:2 328-335.

Mikshis NI, Stepanov AS, Shevchenko OV, and Eremin SA (1999) The intrastrain heterogeneity of the causative agent of anthrax. [Article in Russian] *Zh Mikrobiol Epidemiol Immunobiol.* (3):78-9.

Miyoshi S, Nakazawa H, Kawata K, Tomochika K, Tobe K, and Shinoda S (1998) Characterization of the hemorrhagic reaction caused by Vibrio vulnificus metalloprotease, a member of the thermolysin family. *Infect Immun* 66(10):4851-5.

Moayeri M, Haines D, Young HA, and Leppla SH (2003) Bacillus anthracis lethal toxin induces TNF-alpha-independent hypoxia-mediated toxicity in mice. *J Clin Invest* 112(5):670-82.

Okamoto T, Akaike T, Suga M, Tanase S, Horie H, Miyajima S, Ando M, Ichinose Y, and Maeda H (1997) Activation of human matrix metalloproteinases by various bacterial proteinases. *J. Biol. Chem.* 28;272(9):6059-66.

Park JM, Greten FR, Li Z-W, and Karin M (2002) Macrophage apoptosis by anthrax lethal factor through p38 MAP kinase inhibition. *Science* 2002; 297 2048-2051

Park S, Leppla SH (2000) Optimized production and purification of *Bacillus anthracis* lethal factor. *Protein Exper Purif* 2000; 18:293-302

Pellizzari R, Guidi-Rontani C, Vitale G, Mock M, Montecucco C (1999) Anthrax lethal factor cleaves MKK3 in macrophages and inhibits the LPS/IFN-γ-induced release of NO and TNF-α. *FEBS Lett* 1999; 462:1-2 199-204

Popov SG, Villasmil R, Bernardi J, Grene E, Cardwell J, Wu A, Alibek D, Bailey C, Alibek K (2002a) Lethal Toxin of *Bacillus anthracis* Causes Apoptosis of Macrophages. *Biochem Biopys Res Commun* 2002; 293 349-355

Popov SG, Villasmil R, Bernardi J, Grene E, Cardwell J, Popova T, Wu A, Alibek D, Bailey C, and Alibek K (2002b) Bactericidal Activity of Human Peripheral Blood Mononuclear Cells Is Inhibited by Lethal Toxin of *Bacillus anthracis. FEBS Letters* 2002, 527:211-15

Popov SG, Popova TG Grene E, Klotz F, cardwell J, Bradburne C, jama Y, Maland M, Wells J, Nalca A, Voss T, Bayley C and Alibek K (2004) Systemic Cytokine Response in Murine Anthrax. *Cell Microbiol.* 2004, 6:226-33

Read TD, Peterson SN, Tourasse N, Baillie LW, Paulsen IT, Nelson KE, *et al.* 2003. The genome sequence of Bacillus anthracis Ames and comparison to closely related bacteria. *Nature* 423(6935):81-6.

Read TD, Salzberg SL, Pop M, Shumway M, Umayam L, Jiang L, Holtzapple E, Busch JD, Smith KL, Schupp JM, Solomon D, Keim P, and Fraser CM (2002) Comparative genome sequencing for discovery of novel polymorphisms in Bacillus anthracis. *Science* 296(5575):2028-33.

Sakata Y, Akaike T, Suga M, Ijiri S, Ando M, and Maeda H (1996). Bradykinin generation triggered by Pseudomonas proteases facilitates invasion of the systemic circulation by Pseudomonas aeruginosa. Microbiol Immunol 40(6):415-23.

Serrao KL, Fortenberry JD, Owens ML, Harris FL, Brown LA. (2001) Neutrophils induce apoptosis of lung epithelial cells via release of soluble Fas ligand. *Am J Physiol Lung Cell Mol Physiol* 2001; 280:2 298-305.

Shin YH, Akaike T, Khan MM, Sakata Y, and Maeda H (1996) Further evidence of bradykinin involvement in septic shock: reduction of kinin production in vivo and improved survival in rats by use of polymer tailored SBTI with longer t1/2. *Immunopharmacology.* 33(1-3):369-73

Smith H, and Keppie J (1954) Observations on experimental anthrax: Demonstration on a specific lethal factor produced *in vivo* by *Bacillus anthracis. Nature.* 173:869-70.

Smith SR, Denhardt G, Terminelli C (2002) A role for histamine in cytokine modulation by the adenosine A(3) receptor agonist, 2-Cl-IB-MECA. Eur J Pharmacol 13;457(1):57-69.

Smith H, Keppie J, Stainley JL (1955) The chemical basis of the virulence of *Bacillus anthracis vs.* the specific toxin produced by *B. anthracis in vivo. Br J Exp Pathol* 1955; 36:460-472.

Sodeinde OA, Subrahmanyam YV, Stark K, Quan T, Bao Y, and Goguen JD (1992) A surface protease and the invasive character of plague. *Science.* 258(5084):1004-7

Stepanov AV, Mikshis NI, Eremin SA, and Bolotnikova MF (1999) Contribution of determinants, located in Bacillus anthracis chromosomes, in realizing the pathogenic properties of the pathogen. [Article in Russian] *Mol Gen Mikrobiol Virusol.* (1):20-3.

Supuran CT, Scozzafava A, and Clare BW (2002) Bacterial protease inhibitors. *Med. Res. Rew.* 22(4):329-72.

Tanaka M, Suda T, Takahashi T, Nagata S (1995) Expression of the functional soluble form of human fas ligand in activated lymphocytes. *EMBO J* 1995; 14(6):1129-1135.

Tanaka M, Suda T, Yatomi T, Nakamura N, Nagata S (1997) Lethal effect of recombinant human Fas ligand in mice pretreated with Propionibacterium acnes. *J Immunol* 1997; 158:5 2303-2309.

Turnbull PC, French TA, and Dowsett EG (1977) Severe systemic and pyogenic infections with Bacillus cereus. *Br Med J* 1(6077):1628-9.

Vasconcelos D, Barnewall R, Babin M, Hunt R, Estep J, Nielsen C, Carnes R, and Carney J. 2003. Pathology of inhalation anthrax in cynomolgus monkeys (Macaca fascicularis). *Lab Invest* 83(8):1201-9.

Welkos SL, Keener TJ, Gibbs PH (1986) Differences in susceptibility of inbred mice to Bacillus anthracis. *Infect Immun.* 51(3):795-800.

Wright GG, Hedberg MA, and Slein JB (1954) Studies on immunity in anthrax. III. Elaboration of protective antigen in a chemically-defined, non-protein medium. *J Immunol* 72: 263-69.

Young, N.S., Levin, J., and Prendergast, R.A. (1972) An invertebrate coagulation system activated by endotoxin: evidence for enzymatic mediation. *J Cli. Invest* 51, 1790-1797.

Effective Antiprotease-Antibiotic Treatment of Experimental Anthrax

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Abstract

Inhalation anthrax is characterized by a systemic spread of the challenge agent, *Bacillus anthracis*. It causes severe damage, including multiple hemorrhagic lesions, to all host tissues and organs. It is widely believed that anthrax lethal toxin secreted by proliferating bacteria is a major cause of death, however, the pathology of intoxication in experimental animals is drastically different from that found during the infectious process. In order to close a gap between our understanding of anthrax molecular pathology and the most prominent clinical features of the infectious process we undertook bioinformatic and experimental analyses of potential proteolytic virulence factors of *B. anthracis* distinct from lethal toxin. Our data demonstrate that atoxigenic *B. anthracis* strain delta Ames ($pXO1^-$, $pXO2^-$) cultivated under aerobic conditions secrets a number of metalloprotease virulence factors, including those with hemorrhagic, caseinolytic and collagenolytic activities, belonging to M4 and M9 elastase/thermolysin families. These factors are directly toxic to DBA/2 mice upon intratracheal administration at 0.5 mg/kg and higher doses. Chemical inhibitors of these factors (phosphoramidon and 1, 10-phenanthroline), as well as immune sera against M4 and M9 proteases of *B. anthracis*, were tested in DBA/2 mice challenged with *B. anthracis* (Sterne) spores. These substances demonstrate a substantial protective efficacy in combination with ciprofloxacin therapy initiated as late as 48 h post spore challenge, compared to the antibiotic alone. Our findings suggest a new direction in the development of anthrax therapeutic modalities complementing anti-lethal toxin therapy.

Introduction

Inhalation anthrax is a severe, often fatal disease characterized by systemic spread of the challenge agent, *Bacillus anthracis*, which is capable of causing severe damage to all tissues and organs. Multiple hemorrhagic lesions in the mediastinum, mediastinal lymph nodes, bronchi, lungs, heart, spleen, liver, intestines, kidneys, adrenal glands, and/or central nervous system are typically found upon postmortem examination of patients who succumbed to inhalation anthrax. The most dramatic and potentially life-threatening changes were observed in the vascular system with a diffuse vasculitis extending from moderate sized arteries and veins down to the capillary level. The vasculitis was often associated with vessel destruction, especially of the smallest vessels, and was typically accompanied by massive necrosis in some tissues (1, 13, 45). It is widely believed that anthrax lethal toxin (LT) secreted by proliferating bacteria is a major cause of death in man and in several other susceptible animal species (16). However, the pathology of intoxication in experimental animals is drastically different from that found during the natural infectious process . Recent extensive analyses in mice and rats challenged with a highly purified lethal toxin (6, 29) confirmed earlier observations (20) that toxin activity caused no gross pathology and almost solely manifested in hypoxic liver failure. In addition to lethal toxin, the hemorrhagic and other tissue-damaging factors elaborated by *B. anthracis* could play important virulence-enhancing roles but these factors have not yet been characterized. In fact, early publications using culture filtrates of *B. anthracis* assumed that the observed effects of secreted substances were caused by LT (10, 11). It is also possible that these other factors could themselves exhibit a direct lethal effect.

The capacity of bacteria to cause destruction of tissues, as well as other pathological consequences such as degradation of immunoglobulins, cytokines and complement, release of inflammatory mediators, or activation of host proteolytic enzymes, is attributed to a wide variety of secreted proteases (43), however in the case of *B. anthracis* the proteases of this microorganism, other than lethal factor, have attracted little attention in the scientific literature. The current study aimed to carry out an initial characterization of certain *B. anthracis* proteolytic enzymes and to obtain evidence on their pathogenic role in anthrax.

Complete genome sequences of two virulent anthrax strains are now available (35, 36), in addition to the sequences of two avirulent species from the same family, namely *B. cereus* (18) and *B. subtilis* (24). This information, along with the known sequence motifs characteristic of hundreds of families of proteolytic enzymes (MEROPS database; ref. 3), allows a whole-genome level comparative analysis of all protease genes that are present in genomes of the three species. The evidence presented here shows that metalloprotease (MP) enzymes which act primarily as collagenases are potent proteases secreted by *B. anthracis* in culture. In addition, several closely related thermolysin-like MPs of the M4 family are candidate enzyme capable of causing a hemorrhagic effect similar to thermolysin (EC 3.4.24.27) from *Bacillus*

thermoproteolyticus. Moreover, these MPs are more abundant in both *B. anthracis* and *B. cereus*, compared to the avirulent *B. subtilis*.

B. anthracis strain delta Ames is devoid of both toxigenic plasmids and produces neither lethal nor edema toxins. Nevertheless, we demonstrate that the proteins secreted by this strain are directly lethal to mice upon intra-tracheal administration at doses as low as 0.5 mg/kg. The death of these animals can take place as quickly as a few hours after administration of the proteins.

In comparison, lethal toxin in similar condition is non-lethal. In order to evaluate the role of secreted MPs as virulence factors in the anthrax infectious process we used well-known protease inhibitors (phosphoramidon and 1,10-phenanthroline), as well as polyclonal rabbit antisera raised against peptides representing common motifs of several *B. anthracis* MPs, for treatment of mice challenged with *B. anthracis* (Sterne) spores. The inhibitors alone were ineffective in protecting mice, while a post exposure administration of immune serum to challenged mice demonstrated substantial protective effect (30 to 60%), depending on the dose and specificity of a particular antiserum. Both a strong synergistic enhancement of survival rates (up to 90% protection) and a faster recovery were observed when, in addition to either the inhibitor or the serum therapy, mice received antibiotic treatment, which alone protected only 20% of the mice in the conditions of our experiments. We conclude that secreted proteolytic virulence factors other that lethal toxin play an important role in anthrax and therefore have to be targeted by a combination post-exposure therapy.

MATERIALS AND METHODS

Microbial strains. The non-encapsulated *Bacillus anthracis* strain 34F2 (Sterne) [pXO1+, pXO2-] obtained from the Colorado Serum Company (Boulder, CO) was used in animal challenge experiments. The 50% lethal doses (LD_{50}s) by the inraperitoneal (i.p.) route were established earlier (Popov *et al.*, 2004) and the LD_{50} value for intraperitoneal challenge for DBA2 mice was found to be 3×10^6 spores per mouse. The non-encapsulated, atoxigenic strain of *B.anthracis* (delta Ames) [pXO1-, pXO2-] was kindly provided by Dr. J. Shiloach (National Institutes of Health, Bethesda, MD). *B. cereus* strain ATCC #11778 and *B. subtilis* strain #23857 were purchased from American Type Culture Collection (Manassas, VA).

Mice. Female DBA2 mice (9 weeks old) were obtained from Taconic (Germantown, NY) and were used throughout the study.

Reagents. The following substances were used in this study: ciprofloxacin (ICN Biomedicals, lot no.4913F), phosphoramidon disodium salt, and 1,10-phenanthroline (Sigma, MO), EDTA (GibcoBRL, CA), soybean trypsin inhibitor from Glycine max (Sigma, MO), thermolysin (EC 3.4.24.27) from *Bacillus thermoproteolyticus* (Sigma, MO). The fluorescently labeled casein and collagen type I for determination of proteolytic acivity were from Molecular

Probes (OR). Zymogram gels were from Invitrogen (Carlsbad, CA). Lethal factor (LF) and protective antigen (PA) were from List Biological Laboratories (CA).

Preparation of secreted proteins. Secreted substances were prepared by culturing *B. anthracis* (delta Ames) in LB media overnight. Cells were removed by centrifugation at 8000 g, and the supernatant was sterilized by filtration through 0.22 μm cellulose acetate filtration system (Corning, NY) and further concentrated 50-fold using Amicon Ultra15 centrifugal filter devices (10K cut-off pore size) (Millipore, MA). The proteins were used immediately after preparation or were stored at 4°C for several days. Protein content was determined using Bradford reagent (Bio-Rad) with bovine serum albumin as standard. Slow reduction in the hemorrhagic activity was found upon storage within a week.

Fractionation of culture supernatants. 1 ml of *B.anthracis* culture supernatant (BACS) was loaded onto the size-exclusion Superdex column (25x60, Pharmacia Biotech) and was eluted with PBS (pH 7.4) with a flow rate of 2 ml/min. Fractions of eluate were concentrated to equal volumes using Centricon devices (Millipore, MA) with a 10K cut-off pore size.

Hemorrhages in femoral artery region. Mice were anesthetized by intraperitoneal injection of Avertin (2,2,2 tribromethanol, Aldrich) and 100μl of *B.anthracis* secreted proteins (20 to 100 μg) were subcutaneously (sc) injected into the femoral artery region for observation of hemorrhagic changes after 3 to 15 h. In order to record hemorrhagic changes animals were anesthetized by i.p. injection of Avertin and the fur over the femoral artery region was removed to allow open observation of a 1.5 to 2.5 cm^2 area of skin. It was photographed, and the size of the hemorrhagic spot was measured. In the experiments on the inhibition of hemorrhagic effect the secreted proteins were preincubated with specific antisera or protease inhibitors for 30 min on ice.

Generation of antibodies against B. anthracis MPs. The Invitrogen (CA) custom service was used to obtain rabbit polyclonal sera against peptides conjugated with kallikrein (Table 1). Two animals were immunized by each conjugate. All six rabbit sera had ELISA titers ranging from 100,000 to 200,000. For generation of murine polyclonal antibodies against the M4 protease (BA3442) the C-terminal part of the gene encoding amino acids 248 to 532 was cloned into pTrcHis2 TOPO TA cloning vector (Invitrogen, CA). The recombinant protein containing a 6xHis tag was expressed in *E.coli* and purified using the Ni-NTA resin (Quiagen, CA). Mice were immunized with 50 μg of the protein emulsified in a complete Freund's adjuvant and were given two booster immunizations using an incomplete adjuvant with 2 week intervals. Serum was collected after two weeks since the last boost injection. In the skin hemorrhagic test described above, 30 μl of serum was able to completely suppress the hemorrhage caused by 30 μl of BACS.

Intratracheal delivery of B. anthracis secreted proteins. Mice were anesthetized by i.p. injection of Avertin and a 24G angiogenic catheter (BD Biosciences, CA) was inserted into the

trachea. 50μl of experimental mixture, containing 10 to 100 μg of culture supernatant proteins were slowly injected through the catheter connected to a microsyringe. The angiogenic catheter was removed and animals were left for further observation. The untreated control group received the same volume of phosphate-buffered saline (PBS). A control group of 3 animals was injected with 50 μl of PBS solution of lethal toxin (100μg PA+100μg LF). In all experiments the rate of breathing was recorded every 10 min during the first 3 h following injection, and animals were observed for survival for 7 days.

Treatment of spore-challenged mice. Mice used in all experiments were maintained under proper conditions with a 12-h light/dark cycle in accord with IACUC standards in the animal facility of the Biocon, Inc. (Rockville, MD). Mice received food and water *ad libitum.* Groups of 10 mice were randomly assigned for challenge and were observed for survival and signs of disease. The animals were inoculated i.p. by 1×10^7 spores per mouse of Sterne strain. Treatment (i.p.) with phenanthroline (30 mg/kg), phosphoramidon (10 mg/kg), or rabbit sera (5 or 25 mg/kg) was carried out individually for each substance or in combination with ciprofloxacin at (50mg/kg) once a day started at different time points post spore challenge and continued for 10 days. In all experiments the animals were monitored for survival for at least 12 days after termination of treatment.

Statistical analysis. Kaplain-Meier open-end survival analysis was performed to compare results between treatment groups. Statistical significance was established as $P<0.05$ using log-rank test.

RESULTS

Genomic analysis of B. anthracis secreted proteins as potential virulence factors. In order to evaluate a pathogenic potential attributed to the *B. anthracis* proteins other than known lethal and edema toxins we used a nontoxigenic and nonencapsulated strain of *B. anthracis* (delta Ames), which is a parental Ames strain cured of both plasmids, pXO1 and pXO2. The substances secreted by vegetative *B. anthracis* cells seem to be the most promising candidates, as is the case for many bacterial toxins (43). Analysis of the chromosome sequence of the *B. anthracis* Ames strain revealed a variety of potential virulence-enhancing factors, including collagenases, phospholipases, haemolysins, proteases and other enterotoxins identified based on their sequence homology with pathogenic factors in other bacterial species (35). The *B. cereus* group, which includes *B. anthracis, B. thuringiensis and B. cereus,* has an expanded number of predicted secreted proteins relative to nonpathogenic *B. subtilis* (35). These *B. cereus* group-specific genes represent the ancestral adaptations to a pathogenic lifestyle by the common ancestor, which was quite similar to *B. cereus*. Our attention was attracted to the group of proteases that are encoded on the *B. anthracis* chromosome, shared in common with *B. cereus*

but absent or relatively rare in the genomes of nonpathogenic bacteria. A large number of these proteases fall into clan MA (classified according to the MEROPS system), which among others includes thermolysin-like enzymes of the M4 family. Metalloproteases (MPs) from several bacterial species belonging to this family are capable of causing massive internal hemorrhages and other life-threatening pathologies (28, 30, 37, 38, 43).

Whole genome analyses also indicated collagenolytic proteases of the M9B family as potentially having pathogenic functions. Eleven protease families are present in *B. anthracis* and *B. cereus* but absent in *B. subtilis*. Six of the eleven subfamilies encode MPs. Three of the latter, namely M6, M9B, and M20C subfamilies, are encoded on the bacterial chromosomes. Members of the M6 peptidase family are usually annotated as "immune inhibitors" because in *B. thuringiensis* they can inhibit the insect antibacterial response (25). The M20C peptidase subfamily represents exopeptidases (4) that are the unlikely cause of tissue destruction or internal bleeding.

Based on the above analysis, this study focused on the M4 family thermolysin/elastase-like neutral proteases and the M9 family collagenases as the candidate virulence-enhancing factors of *B. anthracis* Aimes strain. ***Hemorrhagic, caseinolytic and gelatinolytic activities of anthrax proteases.*** The proteins secreted by three *Bacillus* species (*B. anthracis, B. cereus and B. subtilis*) into culture media were prepared by successive steps of inoculation of the culture media with spores, overnight incubation at 37°C, removal of bacterial cells by centrifugation, sterilization of the supernatant by filtration through 0.22 μ filter and further 50-fold concentration using ultrafiltration devices Amicon Ultra 15 (Millipore, MA) with a 10 KDa cutoff size. The SDS-PAGE gel separation (Fig. 1A) demonstrates the protein content in the concentrated *B. anthracis* culture supernatant (designated as BACS) used in our animal tests. Similar procedures were used to prepare culture supernatants for *B. cereus* ATCC #11778 and *B. subtilis* ATCC #23857 (designated BCCS and BSCS, respectively).

The concentrated culture supernatants were tested in mice. Upon subcutaneous administration, mice developed hemorrhages of different intensity within several hours (Fig. 2A, B). BCCS showed the highest activity followed by BACS, while BSCS was completely inactive. Chemical inhibitors such as phosphoramidon (potent chelating inhibitor of thermolysin and other M4 bacterial metallo-endopeptidases , ref. 22), EDTA (specific for a broad range of MPs) and soybean trypsin inhibitor (SBTI, reversible competitive inhibitor of trypsin and other trypsin-like proteases such as chymotrypsin, plasmin and plasma kallikrein, ref 43) effectively abrogated the hemorrhagic affect of BACS (Fig.1C). The murine serum raised against the recombinant protein corresponding to the mature form of the M4-type thermolysin-like neutral protease of *B.anthracis* (gene identification number, BA 3442 according to ref. 35) was also effective in suppressing the hemorrhagic effect in the skin test. In negative control experiments, neither naïve murine serum nor three irrelevant murine sera against *B. anthracis* candidate pathogenic factors, hemolysins O,

A and B (21) showed anti-hemorrhagic activity (data not shown). Additional control experiments demonstrated that under the conditions of our test the hemorrhagic activity of thermolysin from *B. thermophilicus* was detectable in a dose range from 10 to 100 μg, similar to that for BACS. In contrast to BACS, the inhibitors displayed only partial protection in the case of BCCS (Fig.2C). Overall, these results are consistent with the experimental data that *B. anthracis* culture supernatants were less toxic to mice compared to *B. cereus* ones (5, 11).

Caseinase and gelatinase activities of BACS are readily detected by zymography using casein or gelatin (denatured collagen) (Fig.1D, E). A major band of gelatinase activity corresponds to molecular mass of about 100 KDa, whereas a collagenase activity is represented by about 55 KDa proteins. Phenanthroline was able to completely suppress the caseinolytic activity of BACS at concentration of 100 nM (data not shown).

Generation of antibodies against B. anthracis MPs. Obvious complexity of the BACS protein composition prompted us to develop specific means of detection and inhibition of its components. For this purpose several immune sera were raised in mice and rabbits using the antigens listed in Table 1. The sera were used in Western blots of BACS proteins. When the proteins were directly separated in the SDS-PAGE for subsequent transfer to the nitrocellulose membrane, the resulting blots were of low intensity indicative of proteolytic degradation during the electrophoresis (Fig.1A, left lane). In order to avoid this complication the BACS was fractionated according to the molecular masses of its components on the Superdex size exclusion column in the presence of EDTA as a chelating agent. Analysis of the column fractions in SDS-PAGE showed a complex pattern of proteins bands (Fig. 1). Multiple proteins with a broad spectrum of molecular masses seem to be highly associated and migrate through the column as high molecular mass complexes. Several factors, such as the presence of multiple precursor and mature protein forms resulting from specific proteolytic maturation, along with nonspecific proteolytic products, can potentially contribute to the complexity of the fractions' composition. Western blot experiments with column fractions revealed several discrete bands recognized by antibodies (Fig. 1). The M4 proteases are represented by several bands at about 50 KDa, as well as by the bands at about 40 and 20 KDa. These bands probably correspond to different maturation forms of proteases, including the enzymes lacking signal peptides, and mature enzyme forms. The M9 collagenases are detected as a band with a molecular mass of about 98 kDa which is close to the estimated mass of the pro-enzymes, however the major gelatinase enzymatic activity corresponds to the 55 kDa proteins in the BACS.

Acute toxicity of B. anthracis culture supernatants. Although bacterial proteases are well known pathogenic factors, little information is available regarding their acute toxicity. We tested BACS in mice using intratracheal administration into the lungs because hemorrhagic mediastinitis and lung edema typically precede the lethal outcome in late anthrax. Therefore, lung damage may be considered as a probable death-causing factor. Mice were given different doses

of BACS (10 μg to 40 μg of total protein) and were observed daily for lethality. Fig. 3 shows that depending on the dose all mice died within 2 to 3 days, while the highest dose caused 80% mortality on day 1. For histopathological examination mice were given 100 μg of BACS protein. All animals died within 3 to 4 hours. Postmortem harvested lungs revealed focal intraalveolar acute hemorrhage, which was from minimal to moderately severe with no endothelial cell damage or vasculitis, and mild patchy congestion of medium-size blood vessels. There was evidence of focal platelet accumulation located within areas of hemorrhage or within vessels. In a control experiment, lethal toxin at a comparable dose (100 μg LF, 100 μg PA) caused neither mortality nor hemorrhage, and in fact, produced no significant identifiable histopathological changes.

Protection of mice against anthrax using protease inhibitors. Effective suppression of the hemorrhagic as well as the proteolytic activity of BACS with chemical inhibitors prompted us to test their protective effect against *B. anthracis* infection. We have previously reported the successful application of an adjunct therapy against anthrax infection targeting both bacterial multiplication and host response to infection by using a combination of antibiotic and caspase inhibitors (34). The same principle was used in the current study because inhibition of secreted pathogenic factors is not expected to directly interfere with bacterial multiplication and therefore may not be fully protective. In order to target both the bacteria and the proteolytic factors we used a combination therapy where antibiotic administration was complemented by the administration of a protease inhibitor. We were also interested in the efficacy of delayed treatment initiated after certain periods of time following spore challenge. It is a practically relevant scenario because patients generally seek medical help after the onset of symptoms, and in other patients treatment begins after a certain period of time required to confirm the exposure. In addition, a delayed ciprofloxacin therapy in murine model is only partially protective when currently recommended human antibiotic doses (adjusted for body weight) are used in mice (34). It seemed interesting to study if a combination approach could lead to a synergistic enhancement in survival. Two chemical inhibitors were chosen for this study, phosphoramidon and phenanthroline. Phosphoramidon is a potent inhibitor of thermolysin and other bacterial metallo-endopeptidases, and is effective in suppressing the hemorrhagic effect of BACS. It does not inhibit trypsin, papain, chymotrypsin or pepsin and weakly inhibits collagenase. Phenanthroline is a potent chelating inhibitor of M4 MPs, such as pseudolysin, as well as matrix MPs (43).

Results of three independent experiments are presented in Figs. 4 and 5. Mice were challenged intraperitoneally (i.p.) with about $1x10^7$ of *B. anthracis* Sterne spores. Treatment with a single daily dose of ciprofloxacin (50 mg/kg, i.p.) began immediately after challenge, as well as at 24 h or 48 h post challenge, and continued for 10 days. In our conditions the ciprofloxacin treatment initiated immediately after spore challenge was only 70% effective in preventing death. The survival rate after a 24 h delay in antibiotic administration produced a sharp decline to 20% but remained statistically reliable (compared to untreated group, $p=0.015$). After a 48 h delay the

antibiotic was completely ineffective (p=0.23). The inhibitor treatment without antibiotic was not able to improve survival, however the combination of ciprofloxacin with inhibitors displayed a synergistic increase in protection, especially notable in the case of phenanthroline. The group receiving phenanthroline/ciprofloxacin treatment delayed by 24 h, demonstrated 70% protection of animals, compared to only a 20% survival in the group with ciprofloxacin alone (p=0.03 for these groups). The 48 h-delayed regimen resulted in a statistically reliable 30% protection (relative to the untreated spore-challenged group, $p<0.05$), in contrast to ciprofloxacin alone (relative to the untreated spore-challenged group, p=0.23). There is a similar trend in the efficacy of the combination phosporamidon/ciprifloxacin therapy, compared to ciprofloxacin alone, however the observed differences are less reliable ($p>0.05$).

Protection of mice against B. anthracis using anti-protease sera.

As in the experiments with inhibitors, mice were challenged intraperotoneally (i.p.) with about 30 LD50 of *B. anthracis* Sterne spores. Treatment with a single daily dose of ciprofloxacin (50 mg/kg, i.p.) began at 24 h post challenge and continued for 10 days. The immune sera (each pulled from two rabbits) were administered once daily at a concentration of 25 mg/ml (i.p.). The sera displayed substantial differences in their protective effect (Fig. 6). The anti-M4 serum against the epitope(s) of the active center displayed the highest protection (60%), while the anti-collagenase serum (a-M9Coll) protected 30% mice. The anti-M4EP serum behaved similar to the naïve serum. Both latter sera demonstrated no statistically reliable difference in survival, compared to untreated mice (10%, $p>0.05$). A combination treatment with both antibiotic and all studied immune sera, administered at the same dose (25 mg/kg) was synergistic and protected from 80 to 100% mice. A lower serum dose (5 mg/kg) showed a similar pattern of protection, however the effect of combination treatment was reduced to 70%.

Discussion

In order to be highly virulent, any pathogenic microbe is required to possess the means to effectively establish and further propagate the infectious process. Distinct virulence factors may be necessary to fulfill these requirements at different stages of the disease. *B. anthracis* is a recently emerged highly virulent pathogen which acquired two plasmids pXO1 and pXO2 compared to the genetically similar but opportunistic pathogen *B. cereus*. These plasmids encode for the lethal toxin (LT) and capsule genes, respectively (16). Historically, LT was the first anthrax virulence factor discovered capable of causing death in experimental animals (39). This property of LT essentially abrogated further research efforts on discovery of other potential virulence factors, however it has long been known (40) and now seems well established (12) that LT is not especially toxic, and that the histopathology of LT intoxication differs considerably from that found in clinical anthrax infection (6, 29). It is especially notable that postmortum examination of victims from the Sverdlovsk accident and those autopsied following the 2001 U.S. anthrax attacks

(13,16), revealed hemorrhagic thoracic lymphadenitis and necrotizing hemorrhagic mediastinitis in all patients. About half of the Sverdlovsk victims additionally had hemorrhagic meningitis (13). These severe life-critical symptoms had not been noticed in the intoxicated animals. Some data, however, implicate a new role of LT as a disease-establishing virulence factor playing an important immunosuppressive role within alveolar macrophages at the early stages of inhalation anthrax (31). It has been demonstrated that LT causes apoptotic death of macrophages and its inhibition decreased survival of *B. anthracis* spores engulfed by macrophages (33, 34). These data suggest that the major rolls of LT and ET may actually be to create a more immunologically hospitable environment for the pathogen.

While only fragmented data have been reported on the existence of *B. anthracis* chromosome-encoded virulence factors (21, 27, 42), it is well established that *B.cereus* produces a variety of pathogenic determinants, including a necrotizing enterotoxin, an emetic toxin, extracellular proteases, phospholipases and hemolysins (9). *B. cereus* is capable of causing serious and sometimes lethal infections such as sepsis, pneumonia, meningitis, endocarditis, wound and ocular infections, especially in immunocompromized individuals (2, 8, 9, 14, 44). A highly virulent isolate of *B. cereus* has recently been identified which contains a plasmid 99.6% similar to pXO1 (15). This finding is consistent with the point of view that the *B. cereus* genetic background is sufficient for high virulence when it is complemented with an infection-establishing virulence factor, such as LT. Complete sequencing of the *B. anthracis* and *B. cereus* genomes confirmed their close relationship suggested previously (14) and allowed us to suggest new candidate virulence factors for *B. anthracis*, specifically the proteases of the M4 and M9 families. Structurally similar proteolytic factors in other pathogenic microorganisms are known to be involved in inactivation of complement factors (7), cleavage of serum protease inhibitors (17), activation of blood coagulation system (19), invasiveness into the host tissue (41), and development of hemorrhages (28).

We demonstrate here that secreted metalloproteases (MPs) of *B. anthracis* can digest protein substrates such as casein and gelatin in vitro, and can induce a hemorrhagic process in our test subjects, *in vivo*. Both of these activities are inhibited by chemical inhibitors of M4 and M9 MPs, such as EDTA, phosphoramidon and 1,10-phenanthroline. Consistent with this, the hyper-immune mouse serum against M4 family thermolysin/elastase-like enzyme is capable of inhibiting the hemorrhagic effect of BACS (we currently investigate the *in vitro* inhibiting activity of the hyper-immune sera used in this report). The tissue-damaging action of this type of enzymes is well known (43). For example, pseudolysin, an elastase of Pseudomonas aeruginosa, destroys arterial elastic laminae in systemic infection (23) causes lung damage with hemorrhages and necrosis, causes destruction of epithelial cell function, and induces septic shock through activation of the host kinin cascade.

In the present study we used an intratracheal (i.t.) administration to demonstrate that tissue destructive and hemorrhagic properties of BACS could cause a lethal effect at 0.5 to 3 mg/kg doses of total protein (10 to 60 μg per 20g DBA/2 mouse) within a few days or even hours (Fig. 3). Histopathological examination confirmed our observations of life-threatening severe bleeding upon administration of BACS. It has been reported that *P. aeruginosa* elastase induced an immediate lethal shock in guinea pigs upon an i.v. injection at a similar dose of 1.2 mg/kg. Compared to BACS, the LT is non-toxic upon an i.t. administration. There is no mortality in a control group of LT-treated mice (200 μg/mouse, i.t.). Recently, the toxicity of highly purified LT was re-evaluated in BALB/CJ and C57BL/6J mice, and it was found that doses from 5 to 12.5 mg/kg (i.v.) were required for up to 90% mortality in 5 days (29). We conclude that secreted proteins of *B. anthracis*, in addition to LT and ET, have high pathogenic potential and should be considered as important virulence factors.

We have previously suggested a combination antibiotic-antitoxin approach to anthrax therapy and for the first time demonstrated an increased efficacy of ciprofloxacin treatment in a murine model when it was combined with a caspase inhibitor administration (34). Up to now, there has been no other report on any anti-LT treatment during the anthrax infectious process. In the present study we used two new combination therapies, namely the antibiotic-protease inhibitor and the antibiotic-antiserum ones. Both of them also proved beneficial, compared to antibiotic alone. The inhibitors we used to model anthrax therapy (phosphoramidon and phenanthroline) are not considered as LT inhibitors, however both of these substances increased survival during ciprofloxacin therapy, which was initiated at 24 h and 48 h post challenge. The phenanthroline-ciprofloxacin combination administered 24 h post challenge protected 70% of the mice, compared to 20% for antibiotic alone. Late stages of anthrax are especially difficult to treat (16). In our model the 48 h post challenge time approximately correlates with the period of typical overt anthrax symptoms in patients because 30% of mice die within the next 24 h, a situation that is similar to that clinically observed in natural human infection. In these circumstances our therapy with phenanthroline-ciprofloxacin was 30% protective, while ciprofloxacin alone was ineffective. It is worth pointing out that the high extent of protection (70% after 24 h delay) conferred by a combination of antibiotic with phenanthroline argues against the predominant role of LT as a death-causing factor. We have previously reported that in similar experimental conditions the caspase inhibitor YVAD, capable of protecting macrophages against LT-induced apoptosis, improved survival of DBA/2 mice by 30%. This figure corresponds well to the maximal expected contribution of LT to overall lethality based on our current data. One may therefore expect that a triple component therapy, such as ciprofloxacin-phosphoramidon-caspase inhibitor, might be completely protective. Experiments in this direction are in progress. In connection with the question on the role of LT, we also currently investigate a spectrum of neutralizing activity of immune sera used in this study.

Overall our data demonstrate that *B. anthracis* cultivated in culture media secrets a number of proteolytic virulence factors, including those with hemorrhagic, caseinolytic and gelatinolytic activities. These factors in most respects are distinct from LT, including the mode of their expression under aerobic conditions (LT requires bicarbonate for its expression in vitro, ref. 46), their molecular targets, as well as a high virulent potency upon intratracheal administration. Chemical inhibitors of these factors as well as immune sera raised against them in rabbits demonstrate a substantial protective efficacy in combination with antibiotic therapy. Our findings outline a new direction in the development of anthrax therapeutic approaches, and close a substantial gap between the understanding of anthrax molecular pathology and the most prominent clinical features of its infectious process. Complexity of the BACS composition with regard to the number and specificity of proteolytic enzymes suggests a multitude of their potential virulent mechanisms that need to be explored further.

Acknowledgements

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References

1. **Abramova F.A., L.M. Grinberg, O.V. Yampolskaya, and D.H. Walker.** 1993. Pathology of inhalational anthrax in 42 cases from the Sverdlovsk outbreak of 1979. Proc. Natl. Acad. Sci. U S A. 90(6):2291-4.
2. **Akesson A., S.A. Hedstrom, and T. Ripa.** 1991. Bacillus cereus: a significant pathogen in postoperative and post-traumatic wounds on orthopaedic wards. Scand. J. Infect. Dis. 23(1):71-7.
3. **Barrett A.J.** Bioinformatics of proteases in the MEROPS database. 2004. Curr. Opin. Drug Discov. Devel. 7(3):334-41.
4. **Biagini A., and A. Puigserver.** 2001. Sequence analysis of the aminoacylase-1 family. A new proposed signature for metalloexopeptidases. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 128(3):469-81.
5. **Bonventre P.F., and N.J. Eckert.** The biologic activities of Bacillus anthracis and Bacillus cereus culture filtrates. 1963. Am. J. Pathol. Aug;43:201-11.
6. **Cui X., M. Moayeri, Y. Li, X. Li, M. Haley, Y. Fitz, R. Correa-Araujo, S.M. Banks, S.H. Leppla, and P.Q. Eichacker.** 2004. Lethality during continuous anthrax lethal toxin infusion is associated with circulatory shock but not inflammatory cytokine or nitric oxide release in rats. Am. J. Physiol. Regul. Integ.r Comp. Physiol. 286(4):R699-709.
7. **Cleary P.P., U. Prahbu, J.B. Dale, D.E. Wexler, and J. Handley.** Streptococcal C5a peptidase is a highly specific endopeptidase. 1992. Infect. Immun. 60(12):5219-23.
8. **David D.B., Kirkby G.R., and B.A.Noble.** 1994. Bacillus cereus endophthalmitis. Br J Ophthalmol. 78(7):577-80.
9. **Drobniewski F.A.** 1993. Bacillus cereus and related species. Clin. Microbiol. Rev. 6(4):324-38.
10. **Eckert N.J., and P.F. Bonventre.** 1963. In vivo effects of Bacillus anthracis culture filtrates. J. Inf. Dis. 112:226-32.
11. **Ezepchuk Iu.V., E.D. Bobkova, and N.K. Prosvetova** 1969. [On the toxic and various biochemical properties of filtrates of *B. anthracis* and cereus] Zh. Mikrobiol. Epidemiol. Immunobiol. 46(6):19-23 [Article in Russian]

12. **Friedlander A.M.** 2001. Tackling anthrax. Nature 414(6860):160-1.

13. **Grinberg L.M., F.A. Abramova, O.V. Yampolskaya, D.H. Walker, and J.H. Smith.** 2001. Quantitative pathology of inhalational anthrax I: quantitative microscopic findings. Mod. Pathol. 14(5):482-95

14. **Helgason E., O.A. Okstad, D.A. Caugant, H.A. Johansen, A.Fouet, M. Mock, I.Hegna, and J. Kolsto.** 2000. Bacillus anthracis, Bacillus cereus, and Bacillus thuringiensis--one species on the basis of genetic evidence. Appl. Environ. Microbiol. 66(6):2627-30.

15. **Hoffmaster A.R., J. Ravel, D.A. Rasko, G.D. Chapman, M.D. Chute, C.K. Marston, B.K. De, C.T. Sacchi, C. Fitzgerald, L.W. Mayer, M.C. Maiden, F.G. Priest, M. Barker, L. Jiang, R.Z. Cer, J. Rilstone, S.N. Peterson, R.S. Weyant, D.R. Galloway, D. Read, T. Popovic, and C.M. Fraser.** 2004. Identification of anthrax toxin genes in a Bacillus cereus associated with an illness resembling inhalation anthrax. Proc. Natl. Acad. Sci. U S A. 101(22):8449-54.

16. **Inglesby T.V., T. O'Toole, D.A. Henderson, J.G. Bartlett, M.S. Ascher, E. Eitzen, A.M. Friedlander, J. Gerberding, J. Hauer, J. Hughes, J. McDade, M.T. Osterholm, G. Parker, T.M. Perl, P.K. Russell, K. Tonat; Working Group on Civilian Biodefense.** 2002. JAMA. Anthrax as a biological weapon, 2002: updated recommendations for management. 287(17):2236-52.

17. **Ishihara K., T. Miura, H.K. Kuramitsu, and K.Okuda.** 1996. Characterization of the Treponema denticola prtP gene encoding a prolyl-phenylalanine-specific protease (dentilisin).Infect. Immun. 64(12):5178-86.

18. **Ivanova N., A. Sorokin, I. Anderson, N. Galleron, B. Candelon, V. Kapatral, A. Bhattacharyya, G. Reznik, N. Mikhailova, A. Lapidus, L. Chu, M. Mazur, E. Goltsman, N. Larsen, M. D'Souza, T. Walunas, Y. Grechkin, G. Pusch, R. Haselkorn, M. Fonstein, S.D. Ehrlich, R. Overbeek, and N. Kyrpides.** 2003. Genome sequence of Bacillus cereus and comparative analysis with Bacillus anthracis. Nature 423(6935):87-91.

19. **Kaminishi H., H. Hamatake, T. Cho, T. Tamaki, N. Suenaga, T. Fujii, Y. Hagihara, and H. Maeda.** 1994. Activation of blood clotting factors by microbial proteinases. FEMS Microbiol. Lett. 121(3):327-32.

20. **Klein F., J.S. Walker, D.F. Fitzpatrick, R.E. Lincoln, B.G Mahlandt., W.I. Jones Jr, J.P. Dobbs, and K.J. Hendrix.** 1966. Pathophysiology of anthrax. J. Infect. Dis. 116(2):123-38.

21. **Klichko V.I., J. Miller, A. Wu, S.G. Popov, and K. Alibek.** 2003. Anaerobic induction of Bacillus anthracis hemolytic activity. Biochem. Biophys. Res. Commun. 303(3):855-62.

22. **Komiyama T., H. Suda, T. Aoyagi, T. Takeuchi, and H. Umezawa.** 1975. Studies on inhibitory effect of phosphoramidon and its analogs on thermolysin. Arch Biochem Biophys. 171(2):727-31.

23. **Kon Y., H. Tsukada, T. Hasegawa, K. Igarashi, K. Wada, E. Suzuki, M. Arakawa, and F. Gejyo.** 1999. The role of Pseudomonas aeruginosa elastase as a potent inflammatory factor in a rat air pouch inflammation model. FEMS Immunol. Med. Microbiol. 25(3):313-21.

24. **Kunst F., N. Ogasawara, I. Moszer, A.M. Albertini, G. Alloni, V.Azevedo, M.G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S.C. Brignell, S. Bron, S. Brouillet, C.V. Bruschi, B. Caldwell, V. Capuano, N.M. Carter, S.K. Choi, J.J. Codani, I.F. Connerton, A. Danchin, *et al.*** 1997. The complete genome sequence of the gram-positive bacterium Bacillus subtilis. Nature. 390(6657):249-56.

25. **Lovgren A., M. Zhang, A. Engstrom, G. Dalhammar, and R. Landen.** 1990. Molecular characterization of immune inhibitor A, a secreted virulence protease from Bacillus thuringiensis. Mol. Microbiol. 4(12):2137-46.

26. **Maeda H., T. Akaike, Y. Sakata, and K. Maruo.** 1993. Role of bradykinin in microbial infection: enhancement of septicemia by microbial proteases and kinin. Agents Actions Suppl. 42:159-65.

27. **Mikshis N.I., A.S. Stepanov, O.V. Shevchenko, and S.A. Eremin.** 1999. The intrastrain heterogeneity of the causative agent of anthrax. [Article in Russian] Zh Mikrobiol Epidemiol Immunobiol. (3):78-9.

28. **Miyoshi S., H. Nakazawa, K. Kawata, K. Tomochika, K. Tobe, and S. Shinoda.** 1998. Characterization of the hemorrhagic reaction caused by Vibrio vulnificus metalloprotease, a member of the thermolysin family. Infect. Immun. 66(10):4851-5.

29. **Moayeri M., D. Haines, H.A. Young, and S.H. Leppla.** 2003. Bacillus anthracis lethal toxin induces TNF-alpha-independent hypoxia-mediated toxicity in mice. J. Clin. Invest. 112(5):670-82.

30. **Okamoto T., T. Akaike, M. Suga, S. Tanase, H. Horie, S. Miyajima, M. Ando, Y. Ichinose, and H Maeda.** 1997. Activation of human matrix metalloproteinases by various bacterial proteinases. J. Biol. Chem. 28;272(9):6059-66.

31. **Pellizzari R., C. Guidi-Rontani, G. Vitale, M. Mock, and C. Montecucco.** 1999. Anthrax lethal factor cleaves MKK3 in macrophages and inhibits the LPS/IFN gamma-induced release of NO and TNFalpha. FEBS Lett. 462(1-2):199-204.

32. **Popov S.G., R. Villasmil, J. Bernardi, E. Grene, J. Cardwell, A. Wu, D.Alibek, C. Bailey, and K. Alibek.** 2002. Lethal toxin of Bacillus anthracis causes apoptosis of macrophages. Biochem Biophys Res Commun. 293(1):349-55.

33. **Popov S.G., R. Villasmil, J. Bernardi, E. Grene, J. Cardwell, T. Popova, A. Wu, D. Alibek, C. Bailey, and K. Alibek.** 2002. Effect of Bacillus anthracis lethal toxin on human peripheral blood mononuclear cells. FEBS Lett. 527(1-3):211-5.

34. **Popov S.G., T.G. Popova, E. Grene, F. Klotz, J. Cardwell, C. Bradburne, Y. Jama, M. Maland, J. Wells, A. Nalca, T. Voss, C. Bailey, and K. Alibek.** 2004. Systemic cytokine response in murine anthrax. Cell. Microbiol. 6(3): 225-33

35. **Read T.D., S.N. Peterson, N. Tourasse, L.W. Baillie, I.T. Paulsen, K.E. Nelson, *et al.*** 2003. The genome sequence of Bacillus anthracis Ames and comparison to closely related bacteria. Nature. 423(6935):81-6.

36. **Read T.D., S.L. Salzberg, M. Pop, M. Shumway, L. Umayam, L. Jiang, E. Holtzapple, J.D. Busch, K.L. Smith, J.M. Schupp, D. Solomon, P. Keim, and C.M. Fraser.** 2002. Comparative genome sequencing for discovery of novel polymorphisms in Bacillus anthracis. Science. 296(5575):2028-33.

37. **Sakata Y., T. Akaike, M. Suga, S. Ijiri, M. Ando, and H. Maeda** 1996. Bradykinin generation triggered by Pseudomonas proteases facilitates invasion of the systemic circulation by Pseudomonas aeruginosa. Microbiol. Immunol. 40(6):415-23.

38. **Shin Y.H., T. Akaike, M.M. Khan, Y. Sakata, and H. Maeda** 1996. Further evidence of bradykinin involvement in septic shock: reduction of kinin production in vivo and improved survival in rats by use of polymer tailored SBTI with longer t1/2. Immunopharmacology. 33(1-3):369-73.

39. **Smith H., and J. Keppie.** 1954. Observations on experimental anthrax: Demonstration on a specific lethal factor produced *in vivoi* by *Bacillus anthracis*. Nature. 173:869-70.

40. **Smith H., J. Keppie, and J.L. Stanley.** 1955. The chemical basis of the virulence of *Bacillus anthracis*. V. The specific toxin produced by *B. anthracis in vivo*. Br. J. Exp. Pathol. 36:460-72

41. **Sodeinde O.A., Y.V. Subrahmanyam, K. Stark, T. Quan, Y. Bao, and J.D. Goguen.** 1992. A surface protease and the invasive character of plague. Science. 258(5084):1004-7.

42. **Stepanov A.S., N.I.Mikshis, S.A.Eremin, and M.F. Bolotnikova.** 1999. Contribution of determinants, located in Bacillus anthracis chromosomes, in realizing the pathogenic properties of the pathogen. [Article in Russian] Mol Gen Mikrobiol Virusol. (1):20-3.

43. **Supuran C.T., A. Scozzafava, and B.W. Clare** 2002. Bacterial protease inhibitors. Med. Res. Rew. 22(4):329-72.

44. **Turnbull P.C., T.A.French, and E.G.Dowsett.** 1977. Severe systemic and pyogenic infections with Bacillus cereus. Br. Med. J. 1(6077):1628-9.

45. **Vasconcelos D., R. Barnewall, M. Babin, R. Hunt, J. Estep, C. Nielsen, R. Carnes, and J.Carney.** 2003. Pathology of inhalation anthrax in cynomolgus monkeys (Macaca fascicularis). Lab. Invest. 83(8):1201-9.

46. **Wright G.G., M.A. Hedberg, and J.B. Slein** 1954. Studies on immunity in anthrax. III. Elaboration of protective antigen in a chemically-defined, non-protein medium. J. Immunol. 72: 263-69.

Table 1. Sera against *B. anthracis* proteases

Serum #	Protein family	Protein	Gene number	Antigen	Designation
1	M4	Elastase-like neutral protease	BA3442	Recombinant polypeptide corresponding to the fragment 248-532.	M4EL
2	M9	Collagenase	BA0555, BA3299, BA3584	HEFTHYLQGRYEVPGL spanning the region of active center	M9Coll
3	M4	Neutral protease	BA5282, BA0599	DVIGHELTHAVTE spanning the region of active center	M4AC
4	M4	Neutral protease	BA2730	ADYTRGQGIETY distant from the active center	M4EP

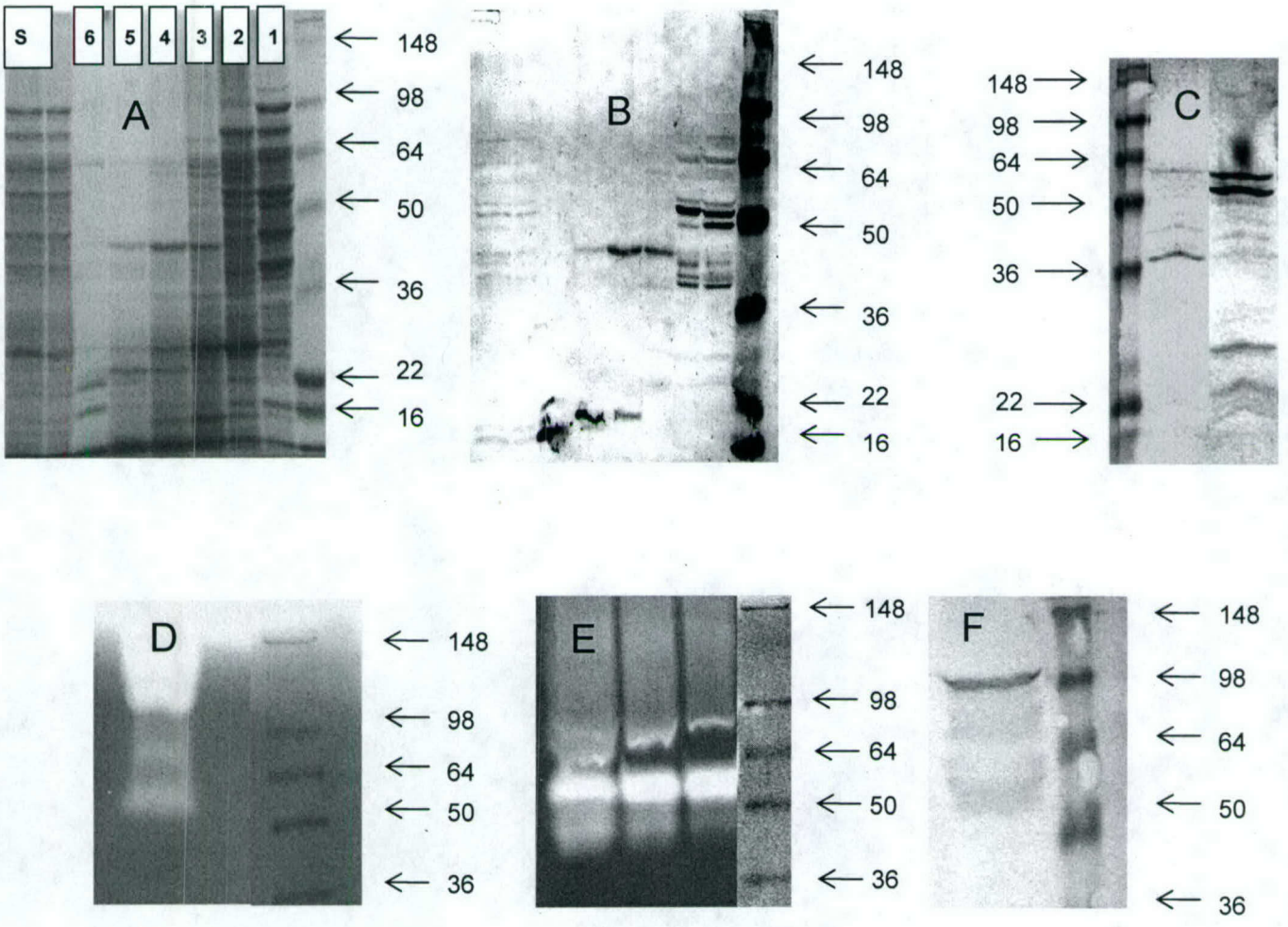
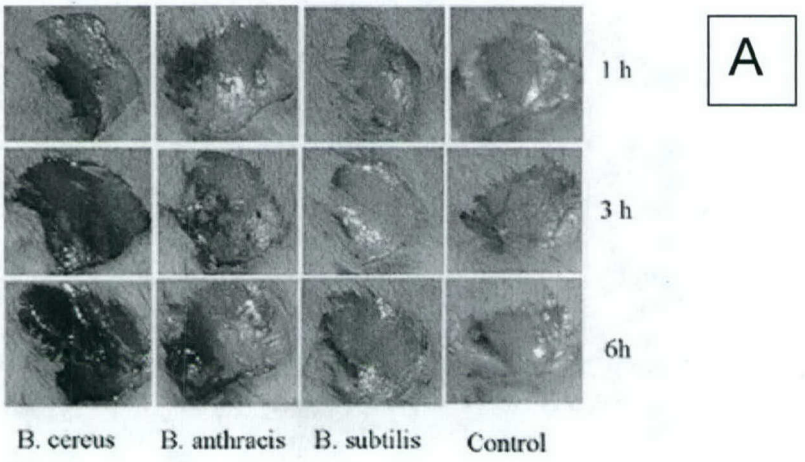


Fig. 1. SDS-PAGE of BACS fractions separated on size exclusion column (A), Western blots: fractions (A) with specific antisera a-M4EL (B), BACS with a-M4AC (C, left panel), BACS with a-M4EP (C, right panel), BACS with a-M9Coll (F), and zymograms of caseinolytic (D) and gelatinolytic (E) activities of BACS. Molecular masses (KDa) of the marker proteins are indicated by arrows. In A, s denoted BACS, and numbers above correspond to column fractions. In E, different amounts of BACS were loaded on a gel (15 μl, 7 μl and 3 μl, from left to right).



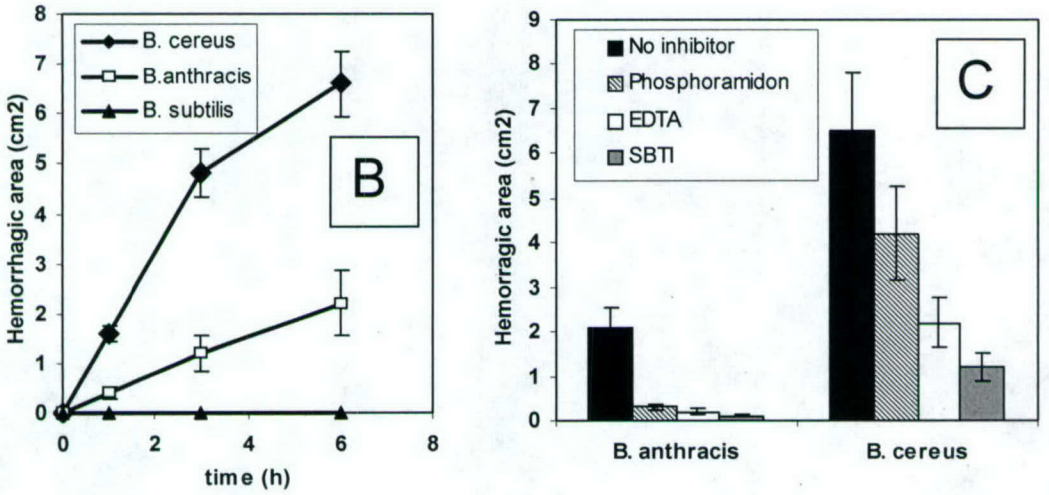


Fig. 2. Hemorrhagic activity of culture supernatants (A), its graphic representation (B) and inhibition with chemical inhibitors (C).

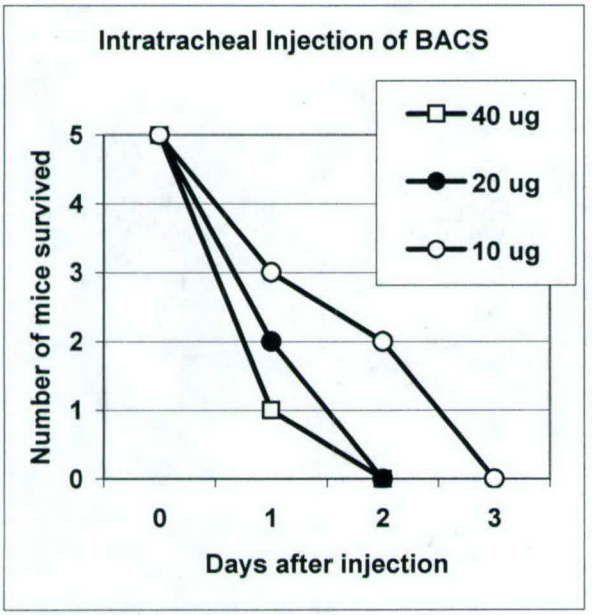
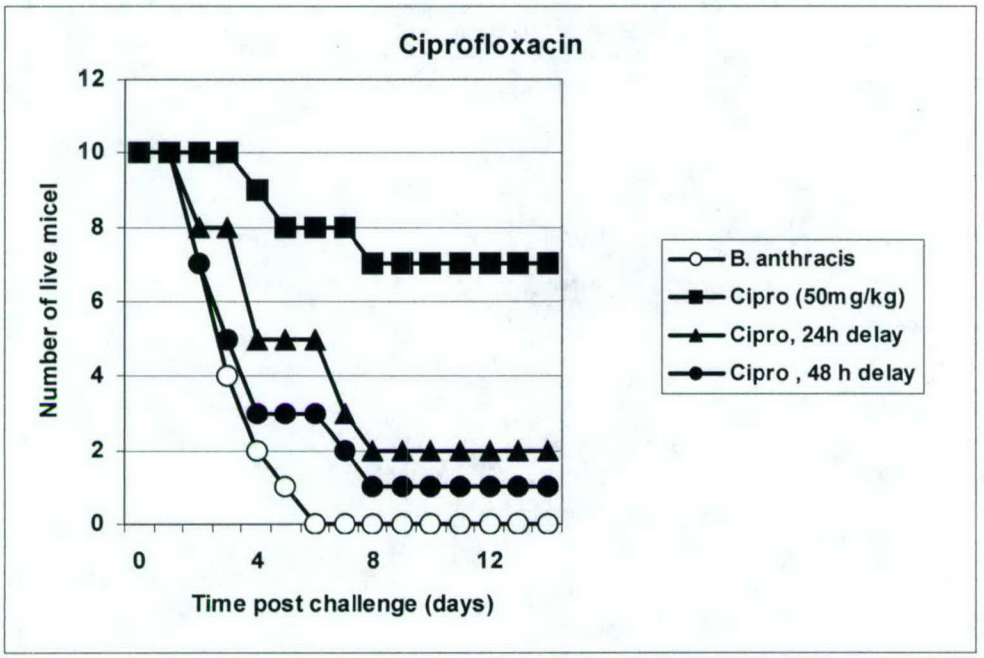


Fig.3. Survival of mice upon intratracheal injection of BACS.



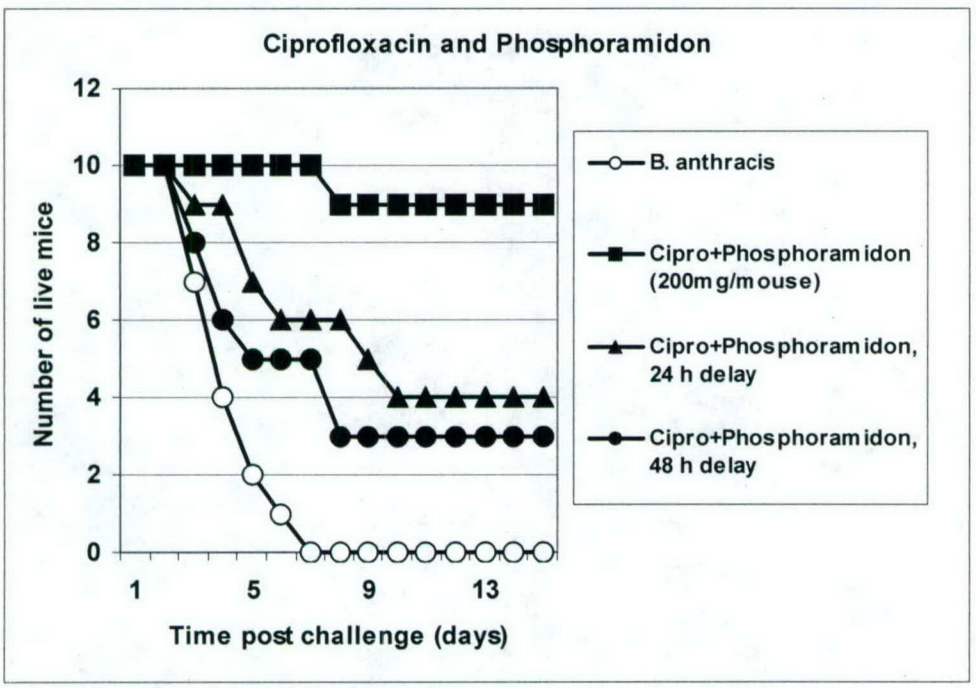
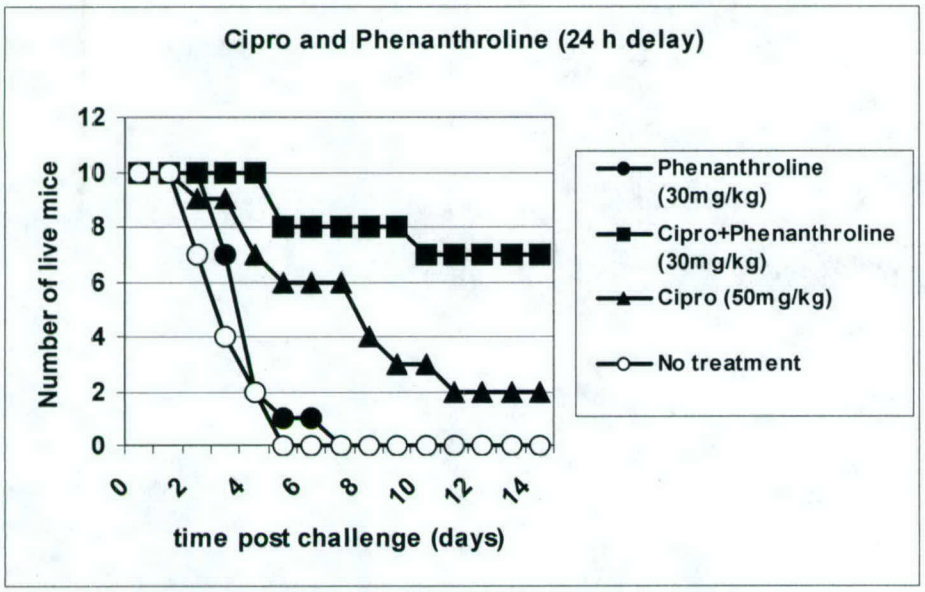


Fig. 4. Protection of mice against *B. anthracis* (Sterne) infection by administration of ciprofloxacin and its combination with phosphoramidon for 10 days beginning 24 h and 48 h post spore challenge.



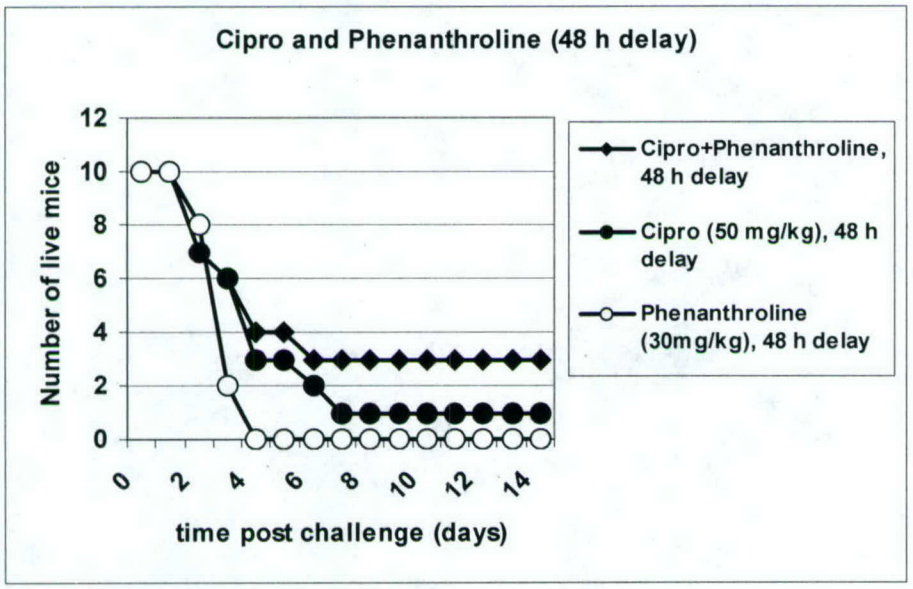
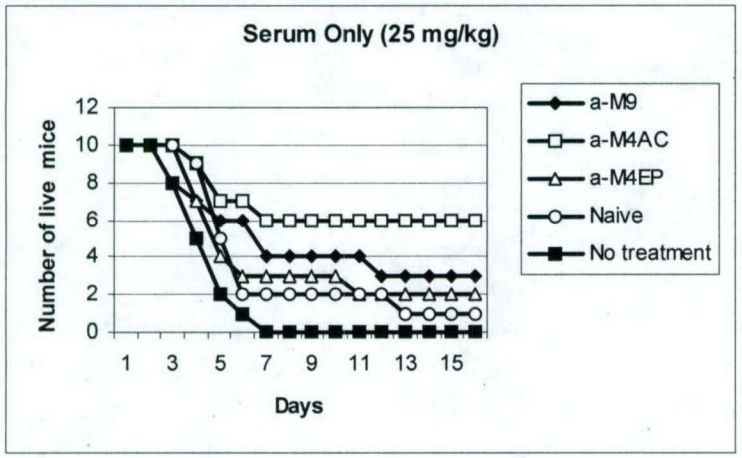
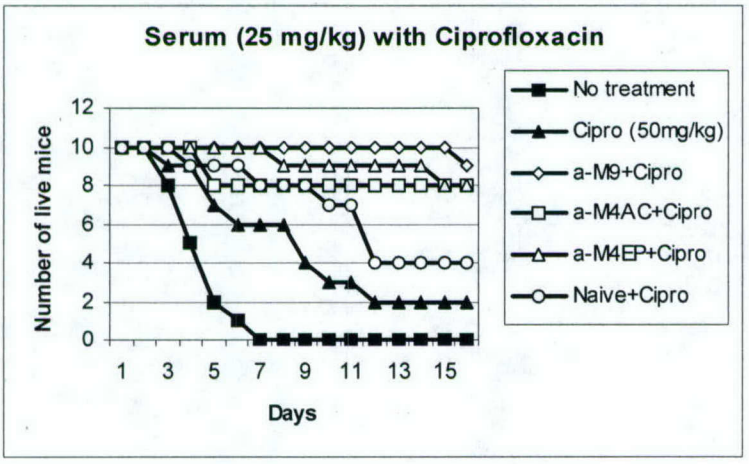


Fig. 5. Protection of mice against *B. anthracis* (Sterne) infection by administration of ciprofloxacin or its combination with phenanthroline for 10 days beginning 24 h and 48 h post spore challenge.





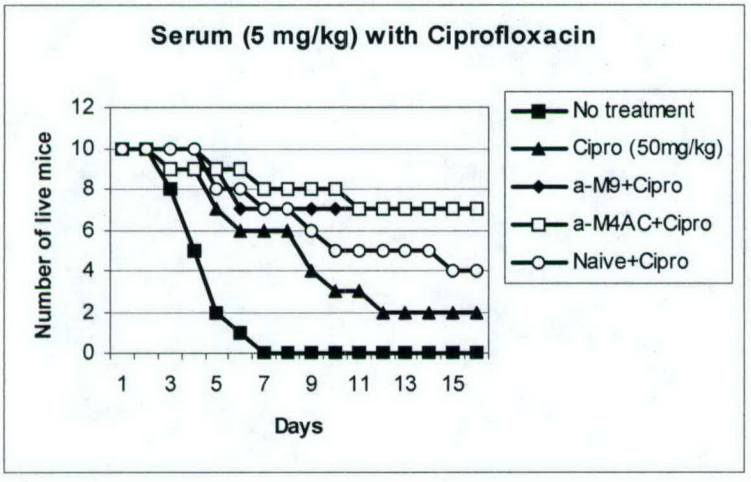


Fig.6. Post exposure efficacy of hyperimmune rabbit sera in mice challenged with *B. anthracis* (Sterne). Treatment with sera and ciprofloxacin was initiated 24 h post exposure and continued for 10 days once daily.

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Bacillus alcalophilus peptidoglycan induces IFN-α-mediated inhibition of vaccinia virus replication

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Abstract

Bacterial products such as cell walls (CW) and peptidoglycan (PGN) are known to activate macrophages and NK cells during microbial infections. In this report, we demonstrated that whole CW and PGN of four Gram-positive bacteria are capable of enhancing the anti-poxviral activity of murine macrophage RAW 264.7 cells. Among the major *Bacillus alcalophilus* CW components, PGN contributes the most to antiviral activity and induces remarkably higher levels of IFN-α. Anti-IFN-α/β antibody, but not anti-IFN-γ, anti-IFN-γ receptor, or anti-IL-12, reversed the PGN-induced inhibition of vaccinia virus replication and reduced nitric oxide (NO) production. Our data thus suggest that PGN induce antiviral activity through IFN-α and to a lesser extent, through NO production.

Keywords: Peptidoglycan; Lipoteichoic acids; IFN-α; NO; Macrophages; Poxviruses

1. Introduction

Innate immunity components such as NK cells, macrophages, and interferons (IFNs) play important roles in the control of early poxvirus infection [1–4]. Macrophages are distributed strategically throughout the body to engage invading pathogens, acting as a first line of defense against microbial attack [4]. In addition to being a professional scavenger, macrophages may serve as an infection target for a virus and can lyse virus-infected cells [3,5]. IFN-α/β and IFN-γ play crucial roles in host anti-poxviral responses [2], as mice lacking IFN-α/β and/or IFN-γ receptors are highly susceptible to poxvirus infections [6,7]. IFNs also regulate immune responses mediated by effector cells such as NK cells and macrophages against viral infections [8,1,9]. For instance, IFN-γ stimulates macrophages to produce nitric oxide (NO) that mediates the inhibition of replication of rhabdoviruses, poxviruses, herpesviruses, and flaviviruses [10–12]. On the other hand, poxviruses encode several cellular homologs of IFN-α/β-binding protein, IFN-γ and TNF-α receptors, and proteins blocking the 2′,5′-oligoadenylate synthetase (OAS)/RNAse L and dsRNA-dependent protein kinase (PKR) pathways [13,14]. Therefore, IFNs, and their immunomodulatory roles in activation of NK cells and macrophages, represent important host defense lines against poxvirus infections.

The cell wall of Gram-positive bacteria consists of a peptidoglycan macromolecule (exoskeleton) with attached accessory molecules such as teichoic acids (TA), lipoteichoic acids (LTA), teichuronic acids, polyphosphates, or carbohydrates [15]. The dry weight of the *Staphylococcus* CW consists of 50–60% PGN and

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30–40% TA and LTA [15,16]. PGN and LTA of Gram-positive bacterial CW are known to act as immunomodulators of NK cells and macrophages [17,18]. The cytolytic activity of macrophages can be regulated by certain bacterial CW components such as lipopolysaccharides (LPS), LTA, PGN or its fragments muramyl peptides to combat intracellular infections [4,17,18]. For example, muramyl dipeptide (MDP), a synthetic muramyl peptide, enhances the lysis of vaccinia virus (VV)-infected target cells [19]. This study was aimed at establishing the role of PGN and LTA from Gram-positive bacteria in macrophage activation and anti-poxviral activity. We provide evidence that *B. alcalophilus* PGN induces the inhibition of VV replication by stimulating murine macrophage RAW 264.7 cells to secret IFN-α.

2. Materials and methods

2.1. Cells and viruses

Murine macrophage cell line RAW 264.7 (ATCC# TIB-71) was maintained in Dulbecco's modified eagle medium (D-MEM/F-12) supplemented with 10% FetalClone I fetal bovine serum (FBS, Hycone), 100 units/ml of penicillin, and 100 μg/ml of streptomycin (GIBCO-BRL). African green monkey kidney BS-C-1 cells (ATCC# CCL-26) and human cervical adenocarcinoma HeLa cells (ATCC# CTL-2.2) were maintained in EMEM medium supplemented with 10% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin (GIBCO-BRL). VV (strain WR) was purchased from ATCC (VR-119), propagated in HeLa cells, and titrated on BS-C-1 cells as previously described [20,21].

2.2. Reagents and antibodies

Recombinant murine IFN-γ was purchased from BioSource International, Inc. Sodium nitrate, α-Amylase, DNase, RNase, alkaline phosphatase, and trypsin were purchased from Sigma–Alrich. Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthylenediamine, 5% H_3PO_4) was purchased from ALEXIS® Biochemicals. All tissue culture media were purchased from GILBCO-BRL. Neutralizing antibodies specific to murine IFN-γ receptor [22], IFN-γ, IFN-α/β, or IL-12 were purchased from R&D Systems. The 50% neutralization doses (ND_{50}) of these antibodies are as follows: hamster anti-IFN-γ R2, 0.2–1 μg/ml in the presence of 8.43 IU/ml of rmIFN-; rat anti-IFN-γ, 0.05–0.15 μg/ml in the presence of 1.0 ng/ml of rmIFN-γ ; and goat anti-mouse IL-12, 0.01–0.02 μg/ml in the presence of 0.3 ng/ml of rmIL-12. Sheep polyclonal anti-mouse IFN-α/β antibody (2×10^5 NU/ml) was purchased from BioSource International [23]. One neutralization unit (NU) is defined as the amount of antiserum required to neutralize 50% of 1 unit of mouse IFN-α/β. All chemicals used for CW fractionations were purchased from Fisher Scientific or VWR Scientific Products Corporation.

2.3. Preparations of bacteria cell walls, PGN, and LTA

Bacteria *Arthrobacter histidinolovorans* (Adams, ATCC# 11442), *Enterococcus faecium* (Orla-Jensen, ATCC# 882), *Macrococcus caseolyticus* (Evans, ATCC# 51834), and *Bacillus alcalophilus* Vedder (ATCC# 27647) were grown following ATCC's recommendations. Bacterial CW, PGN, and LTA were prepared following procedures adapted from those previously published [24,25]. In brief, bacterial cultures were collected by centrifugation (25,000*g* for 20 min). The pellet (150 mg of wet weight) was then subjected to 3–4 cycles of boiling (4% SDS for 15–30 min) and centrifugation, resuspended in 30 ml of 2 M NaCl, centrifuged, washed with pyrogen-free distilled water, and centrifuged. The pellets were freeze dried, resuspended in pyrogen-free distilled water to a final concentration of 1 mg/ml, and stored at −80 °C until used.

PGN was isolated from lyophilized CW following treatment with enzymes and hydrofluoric acid. Thirty milligrams of lyophilized CW were resuspended in 1 ml of 0.1 M Tris–HCl (pH 7.5), treated with α-Amylase (100 μg/ml) for 2 h, DNase and RNase (each at 100 μg/ml) for 2 h, and trypsin (100 μg/ml in 10 mM $CaCl_2$) for 16 h. After boiling for 15 min in 1% SDS, the samples were centrifuged at 40,000*g* for 15 min. The pellet was washed three times with distilled water, once with 8 M LiCl, centrifuged, washed four times with distilled water, and dried. Five milligrams of dry pellet were resuspended in 1 ml of hydroflouric acid (49% w/v), incubated for 48 h at 4 °C, washed four times with distilled water, resuspended in 0.1 M Tris–HCl (pH 7.5), washed until pH was neutral. Following a centrifugation at 30,000*g* for 30 min, the pellet was treated with alkaline phosphatase (250 μg/ml) in 0.1 M $(NH_4)_2CO_3$ for 16 h at 37 °C. After boiling for 5 min and washing twice with sterile distilled water, PGN was resuspended in sterile distilled water and stored at −20 °C.

LTA was purified by resuspending a defrosted aliquot of bacteria in 0.1 M sodium acetate (pH 4.5) to a final concentration of 800 mg/ml, then mixing with equal volume of *n*-butanol for 30 min at room temperature. Following a centrifugation at 13,000*g* for 20 min, the aqueous phase was lyophilized, resuspended in chromatography start buffer (15% *n*-propanol in 0.1 M ammonium acetate, pH 4.7), then centrifuged at 45,000*g* for 15 min. The supernatants were fractionated by hydrophobic interaction chromatography (HIC) on an octyl-Sepharose column (FPLC). The fractions were eluted with increasing concentrations of *n*-propanol (0–60%) in 0.1 M ammonium acetate buffer. LTA preparations were quantified by measuring the organic phosphorus as previously described [26].

An important practical consideration in preparing and testing PGN or LTA is to avoid contamination by LPS from Gram-negative bacteria, which share most of the biological activities of CW components. Therefore, the absence of Gram-negative endotoxin in all the above preparations were confirmed using QCL-1000® Limulus Amebocyte Lysate (BioWhittaker) [27,28]. The DNA and protein contaminations in the above preparations were less than 1% and 3%, respectively.

2.4. Nitric oxide assay

The NO assay was performed by Griess reaction as previously described [29,30]. Briefly, RAW 264.7 cells were seeded in a 96-well plate (2×10^5 per well) and allowed to attach for a minimum of 4 h prior to use. Bacterial CW (1–10 μg/ml), PGN (1–30 μg/ml), LTA (10 μg/ml), or IFN-γ (100 IU/ml) was added to activate cells, and the cells were incubated at 37 °C. Following a 20-h incubation, the cell supernatant (100 μl per well) was mixed with an equal volume of Griess reagent; and absorbance at 550 nm (OD_{550}) was read following an ($\geqslant 10$ min) incubation at room temperature. NO_2^- concentrations were determined by comparing them with a standard curve of sodium nitrate.

2.5. Antiviral assay

The anti-poxvirus activity of macrophages was carried out following a procedure previously described [12,30]. Raw 264.7 cells (2×10^5/well) were seeded in a 96-well plate (2×10^5 per well) and allowed to attach for at least 4 h prior to use. To activate cells, CW (1–10 μg/ml), PGN (1–30 μg/ml), LTA (10 μg/ml), or IFN-γ (100 IU/ml) was added to the medium, and the cells were incubated for 20 h at 37 °C. Human 293 cells were grown overnight and infected with VV at a multiplicity of infection (MOI) of 1 pfu/cell, allowing to absorb for 1 h. The infected cells were washed twice with PBS, detached, and counted. After washing the RAW 264.7 cells twice with PBS, the activated cells were co-cultured with 10^4 VV-infected 293 cells for 16 h. The virus titers in the co-cultures were measured by plaque assay on BS-C-1 cells following three cycles of freezing/thawing [21].

2.6. Antibody neutralization assay

RAW 264.7 cells were seeded at a density of 1×10^5/well in 96-well plates 24 h prior to use. In the antibody neutralization assay, neutralizing anti-IFN-γ, anti-IFN-γ R2, anti-IFN-α/β, or anti-IL-12 antibody at various dilutions was added into the cell culture media 1 h before the addition of PGN (10 μg/ml) and the culture was incubated for 20 h. The same antibody was also included in the co-culture of RAW 264.7 and VV-infected 293 cells for 16–18 h. The rest of the procedure in the assay remained the same as described above.

2.7. Cytokine ELISA

The murine IFN-α and TNF-α secreted to the cell culture supernatant were measured in triplicate by an antibody-sandwich IFN-α ELISA kit (PBL Biomedical Laboratories) and a BD OptEIA™ ELISA set (BD PharMingen), respectively, following the manufacturer's instructions. The detection limits for IFN-α and TNF-α were 12.5 and 15.6 pg/ml, respectively.

2.8. Statistical analysis

Data are expressed as mean ± SD and were compared among groups by Student's *t*-test. A value of $p < 0.05$ was considered to be significant.

3. Results

3.1. In vitro antiviral assays

Antiviral activity of macrophages has previously been categorized as either intrinsic or extrinsic [3,31]. Intrinsic activity refers to the outcome of virus replication within the macrophages, which is important for the establishment of viral infections within macrophages. Extrinsic antiviral activity is defined as the ability to inactivate extracellular viruses or to inhibit the replication of viruses in surrounding susceptible bystander cells. Extrinsic activity is more important during infection, as viruses produced by bystander cells can mediate the spread of viral infection in the body [31]. The extrinsic antiviral activity has been demonstrated for several viruses including poxviruses, and may be a property of activated macrophages [4]. Therefore, we used a published extrinsic assay [12,30] to evaluate the antiviral activity of activated macrophages. The assay involves activation of murine macrophage RAW 264.7 cells with cytokine, CW, or a CW component, infection of human 293 target cells, co-culture of activated macrophages and infected target cells, and titration of the VV titers in the mixture. This assay has been shown to be reproducible and reliable by several other groups and us. Cell–cell contact is apparently required for transferring the antiviral state, as separation of VV-infected 293 (target) and RAW 264.7 (effector) cells by semipermeable membrane abrogates this antiviral effect [12].

3.2. CW and PGN preparations of Gram-positive bacterial induce inhibition of VV replication

RAW 264.7 cells respond well to IFN-γ and/or LPS stimulation for NO production. It has been demon-

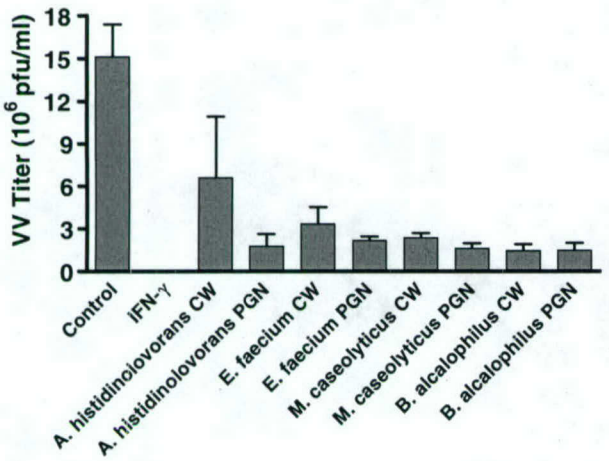

Fig. 1. Effects of bacterial CW and PGN on the inhibition of VV replication. RAW 264.7 cells were activated with indicated bacteria CW (10 μg/ml), PGN (10 μg/ml), or IFN-γ (100 IU/ml) for 20 h. The effector cells were co-cultured with human 293 target cells infected with VV (MOI = 1) for 16 h. The virus titers of the mixtures were measured by plaque assay and expressed as the means of three separate experiments, each containing triplicate samples. Error bars represent standard error of means.

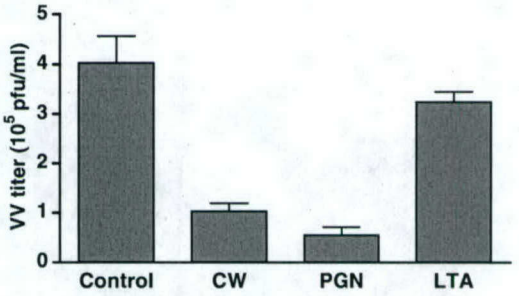

Fig. 2. Effects of CW, PGN, or LTA of *B. alcalophilus* on the inhibition of vaccinia virus replication. RAW 264.7 cells were activated with CW (1 μg/ml), PGN (10 μg/ml), or LTA (10 μg/ml) of *B. alcalophilus* for 20 h, then co-cultured with VV-infected 293 cells (MOI = 1) for 16 h. The virus titers of the mixtures were measured by plaque assay and expressed as the means expressed as means ± SD for triplicate samples. Values represent data from two independent experiments.

strated that IFN-γ is capable of enhancing the antiviral activity of macrophage cells. Pretreatment of RAW 264.7 cells with IFN-γ led to an 85–95% reduction in VV titer (Fig. 1). VV titers in samples treated with whole CW from *A. histidinolovorans*, *E. faecium*, *M. caseolyticus*, and *B. alcalophilus* were reduced to 44%, 22%, 16%, and 12% of that in the control, respectively (Fig. 1). VV titers in samples treated with PGN preparations from *A. histidinolovorans*, *E. faecium*, *M. caseolyticus*, and *B. alcalophilus* were reduced to 12%, 14%, 11%, and 10% of that in the control, respectively (Fig. 1). For each bacterial species, PGN is more or equally effective than its cognate CW in reducing VV replication.

3.3. PGN, but not LTA, is responsible for the antiviral activity

To identify the CW components responsible for the antiviral activity, we examined the effects of the major *B. alcalophilus* CW components, PGN, and LTA, on the inhibition of VV replication in the same assay. Pretreatment of RAW 264.7 cells with CW and PGN reduced VV titers to 25% (ranging 10–25%) and 14% of that in the control (Fig. 2). In contrast, LTA treatment led to a 20% reduction in VV titer compared to that in the control. LTAs from *A. histidinolovorans*, *E. faecium*, and *M. caseolyticus* had no significant effect on VV replication (data not shown). Our data thus indicated that PGN, but not LTA, of Gram-positive bacteria induces antiviral activity of macrophage cells.

We next examined the dose effect of *B. alcalophilus* PGN on the antiviral and NO induction by macrophage cells. PGN within the test range (1–30 μg/ml) reduced VV titer to 10–20% of that in the control (Table 1). Based on these results, we concluded that *B. alcalophilus* PGN may function as a more or equally effective antiviral inducer of murine macrophages compared to sample treated with CW. This is supported by our NO stimulation data (Table 1), showing that PGN, in the test range, induced slightly higher levels of NO than the CW treated sample did.

Table 1
Effect of *B. alcalophilus* PGN on antiviral activity, NO induction, and TNF-α release by macrophage RAW 264.7 cells

Dose (μg/ml)	VV titer (10^5 pfu/ml)	NO_2^- (μM)	TNF-α (ng/ml)
Control	2.21 ± 0.21	0	0.6 ± 0.2
PGN, 1	0.23 ± 0.06	11.9 ± 2.7	4.2 ± 0.7
PGN, 3	0.45 ± 0.22	9.7 ± 0.9	4.0 ± 0.2
PGN, 10	0.27 ± 0.12	8.7 ± 0.9	5.5 ± 0.2
PGN, 30	0.28 ± 0.13	10.3 ± 0.8	3.7 ± 0.7
CW, 1	0.83 ± 0.11	6.9 ± 0.7	3.8 ± 0.1

RAW 264 cells were activated by *B. alcalophilus* CW, PGN at indicated concentrations, or PBS (control) for 20 h. Supernatants were assayed for NO by Griess reaction and TNF-α by ELISA as described in Section 2. The effector cells were cocultured with human 293 target cells infected with VV (MOI = 1) for 16 h. The mixtures were subjected to three cycles of freezing/thawing and viruses were titrated by plaque assay. All the results were expressed as means ± SD of triplicate samples.

3.4. B. alcalophilus *PGN enhances secretion of IFN-α*

To probe the mechanisms by which CW components mediate inhibition of VV by macrophages, we determined the levels of murine IFN-α in the media of macrophage cells activated with *B. alcalophilus* CW, PGN, or LTA using ELISA kit. The levels of IFN-α from the PGN-activated sample were 4.5-fold of that

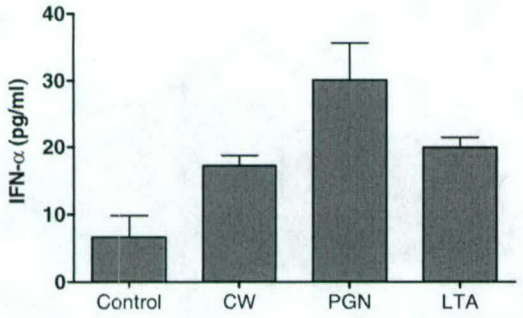

Fig. 3. *B. alcalophilus* PGN induces IFN-α production in RAW 264.7 cells RAW 264.7 cells were activated with IFN-γ (10 U/ml), CW (1 μg/ml), PGN (10 μg/ml), or LTA (10 μg/ml) of *B. alcalophilus* for 20 h, then co-cultured with VV-infected 293 cells (MOI = 1) for 16 h. The levels of IFN-α in the co-culture supernatants were measured by ELISA kit. The results were expressed as means ± SD for triplicate samples. Values represent data from two independent experiments.

from the control, 1.5- and 1.8-folds of those from the LTA- and CW-activated samples, respectively (Fig. 3).

3.5. Anti-IFN-α/β, but not anti-IFN-α or anti-IL-12, antibody reduced NO production and restored VV replication

To identify the endogenous cytokines responsible for the PGN-induced antiviral activity, we conducted an antibody neutralization assay. Macrophage produced IFN-α/β have been shown to mediate the inhibition of VV replication in a similar assay system [30]. IFN-γ is the most potent activator of macrophages. Although T lymphocytes and NK cells are the major producers of IFN-γ, recent studies have shown that IFN-γ is constitutively expressed in resting mouse peritoneal macrophages (PM), LPS- or IFN-γ-stimulated PM [32,33]. The antiviral assay system used in this study is very sensitive to IFN-γ-induced, NO-mediated anti-poxviral activity. Based on the above considerations, we conducted antibody neutralization experiments to examine if PGN stimulates macrophage IFN-γ or its inducer, IL-12, which subsequently induces NO-mediated antiviral activity. The presence of anti-IFN-γ, anti-IFN-γ R, or anti-IL-12 antibody had no significant effect on either the VV replication (Fig. 4(a)) or NO production (Fig. 4(b)). In contrast, anti-IFN-α/β antibody restored the VV replication to 82% of that in control (Fig. 4(a)) and reduced NO production to two thirds of that in the control (Fig. 4(b)). Although high levels of TNF-α were detected in the PGN-treated samples (Table 1), our previous study showed that anti-TNF-α antibody had no effect on VV replication or NO production in this assay system [30]. A 10-fold increase in the concentrations of these antibodies had no significant effect on VV replication or NO production (data not shown).

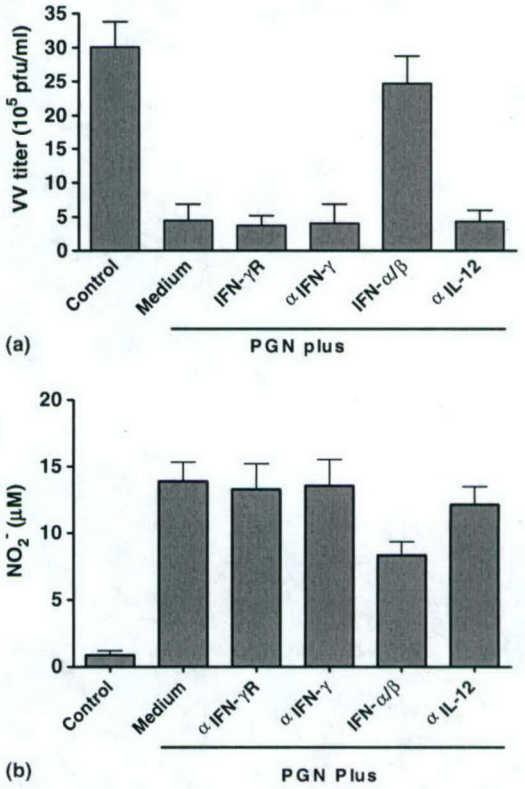

Fig. 4. (a) Effect of neutralizing antibodies on the inhibition of VV replication induced by *B. alcalophilus* PGN. Cells were activated with PGN (10 μg/ml) in the absence or presence of anti-IFN-γ R (5 μg/ml), anti-IFN-γ (5 μg/ml), anti-IFN-α/β (1000 NU/ml), or anti-IL-12 (10 μg/ml) for 20 h, then co-cultured with VV-infected 293 cells (MOI = 1) for 16 h in the presence of the same antibodies. The virus titers of the mixtures were measured by plaque assay and expressed as the means ± SD for triplicate samples. Values represent data from two independent experiments. (b) Effect of antibodies on NO production induced by *B. alcalophilus* PGN. RAW 264.7 cells were activated with PGN (10 μg/ml) in the absence or presence of anti-IFN-γR (5 μg/ml), anti-IFN-γ (5 μg/ml), anti-IFN-α/β (1000 NU/ml), or anti-IL-12 (10 μg/ml) antibody for 20 h. The NO levels in the culture supernatants were measured by Griess assay and expressed as means ± SD for triplicate samples. Values represent data from two independent experiments.

4. Discussion

We examined the effects of CW, PGN, and LTA of Gram-positive bacteria on the extrinsic antiviral activity of macrophages. We found no significant dose response in *B. alcalophilus* PGN-induced inhibition of VV replication (10–20% of that in control), NO production (8.7–11.9 μM), and TNF-α release (3.7–5.5 ng/ml) (Table 1). Nevertheless, PGN is more potent than its cognate CW in its ability to enhance the antiviral activity. The macrophage-mediated antiviral activity has been demonstrated in herpesviruses, vesicular stomatitis virus (VSV), and Venezuelan equine encephalitis virus (VEEV) [34–36]. The mechanisms of this suppression are not fully

understood; however, there is an apparent difference among the mechanisms of host resistance to infections of enveloped viruses [37]. We provide evidence that *B. alcalophilus* PGN induces inhibition of VV replication and IFN-α secretion. Based on our preliminary data and literature analysis, we propose that bacterial PGN may mediate the antiviral activity of macrophages by the following mechanisms.

IFN-α/β induced by PGN, and possibly by VV infection, may enhance macrophage-mediated lysis of infected cells. Several lines of evidence support this idea. Firstly, macrophages can lyse herpesvirus-infected cells in the absence of virus-specific antibody [36,38,39]; this extrinsic antiviral activity has been attributed to the action of interferon [34,35]. Administration of *Nocardia opaca* PGN to mice had a remarkable stimulatory effect upon the cytolytic activity of peritoneal cells possibly mediated through IFN-α/β [40]. Secondly, murine macrophages can also lyse VSV- or VEEV-infected target cells; this macrophage-mediated lysis of infected cells can be enhanced by preexposure to IFN-α/β, IFN-γ or LPS [36]. Thirdly, *Staphylococcus aureus* PGN induced IFN-α/β production by mouse splenocytes after 24 activation, and stimulated the phagocytosis of peritoneal exudate macrophages [41]. Fourthly, we found that PGN enhances the IFN-α secretion in macrophage cells (15–20 pg/ml) and the coculture of macrophages and VV-infected target cells (25–35 pg/ml, Fig. 3). A weak band of IFN-β mRNA was observed in *B. alcalophilus* PGN-activated macrophages (data not shown). IFN-α/β are relatively species specific, being inactive or partly active in human target cells. Therefore, our assays, which used human 293 cells as a target, might have underestimated the direct antiviral effect of IFN-α/β. The direct antiviral effect of IFN-α/β, in which both target and macrophage IFN-α/β are from the same species, is anticipated in vivo.

Alternatively, PGN may directly, or indirectly through IFN-α/β, induce macrophage production of NO, thereby mediating the inhibition of VV replication. NO is known to mediate the inhibition of VV replication by blocking viral DNA replication and late protein synthesis [12]. Gram-positive bacterial PGN has been shown to activate murine macrophages to release NO [42,43]. Depletion of endogeneous IFN-α/β with antibody partly reduced PGN-induced NO production and largely restored antiviral activity (Fig. 4), suggesting that this NO may play a less important role than IFN-α/β do in observed antiviral activity. Therefore, IFN-α together with NO could account for the PGN-induced antiviral activity. Although macrophages have been shown to produce IFN-γ in response to LPS or *Mycobacterium* infection [32,33,44], our Ab depletion data suggest that IFN-γ may not play a role in the PGN-induced antiviral activity.

Effective macrophage activation drugs are needed due to the alarming emergence of antibiotic resistant bacteria and the increasing number of patients with immunodeficiency [45]. Administration of MDP or their analogs can also halt potentially lethal infections of several viruses including VV [19,45–48], indicating that macrophage activation may play a role within the host defense against viral infections. However, the in vivo anti-poxviral role of CW and PGN remain to be established, as intraperitoneal administration of the *B. alcalophilus* CW or PGN failed to protect mice against lethal vaccinia infections (data not shown). Given that it accounts for more than 50% of the CW mass, PGN may play a major role in CW-induced macrophage activation [49]. Although our study did not address the mechanisms by which PGN induces IFN-α and antiviral activity, TLR-2 is likely to be involved. TLR-2-deficient mice failed to respond to *S. aureus* PGN [49]. Our preliminary data showed that *B. alcalophilus* PGN is capable of causing a 15-fold induction of the NF-κB inducible reporter gene (SEAP) expression in the HEK293T cells transiently expressing human TLR2, but not TLR1 or TLR6 (data not shown). In vivo, PGN-induced IFN-α/β as well as CW components may also enhance the cytolytic activity of NK cells against infected target cells, resulting in premature replication and/or assembly of virus particles. Consistent with this idea, NK cells and IFN-α/β have been shown to be required for genetic resistance to lethal infection with ectromelia virus [1]. *S. aureus* and *Micrococcus lysodeikticus* PGN have been shown to elicit the NK populations due to IFN-β [50].

In conclusion, we provided evidence that *B. alcalophilus* PGN induces IFN-α-mediated inhibition of poxvirus replication. NO, to a lesser extent, may also contribute to this antiviral activity of macrophages. PGN, but not LTA, from the four Gram-positive bacteria including *B. alcalophilus* may play a major role in the antiviral activation of macrophages. The potential contribution of TLR2 in the PGN-induced antiviral activity is under investigation.

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References

[1] Jacoby, R.O., Bhatt, P.N. and Brownstein, D.G. (1989) Evidence that NK cells and interferon are required for genetic resistance to lethal infection with ectromelia virus. Arch. Virol. 108, 49–58.

[2] Biron, C.A. and Sen, G.C. (2001) Interferons and other cytokines. In: Fundamental Virology (Knipe, D.M. and Howley, P.M., Eds.), pp. 321–352. Lippincott William & Wilkings, Philadelphia.

[3] Morahan, P.S., Connor, J.R. and Leary, K.R. (1985) Viruses and the versatile macrophage. Br. Med. Bull. 41, 15–21.
[4] Herbein, G. (1997) Cytokines, viruses and macrophages: an interactive network. An immune dysregulation involving the members of the tumor necrosis factor (TNF) receptor superfamily could be critical in AIDS pathogenesis. Pathol. Biol. (Paris) 45, 115–125.
[5] Keller, F., Wild, M.T. and Kirn, A. (1985) In vitro antiviral properties of endotoxin-activated rat Kupffer cells. J. Leukoc. Biol. 38, 293–303.
[6] van den Broek, M.F., Muller, U., Huang, S., Zinkernagel, R.M. and Aguet, M. (1995) Immune defence in mice lacking type I and/or type II interferon receptors. Immunol. Rev. 148, 5–18.
[7] Huang, S., Hendriks, W., Althage, A., Hemmi, S., Bluethmann, H., Kamijo, R., Vilcek, J., Zinkernagel, R.M. and Aguet, M. (1993) Immune response in mice that lack the interferon-gamma receptor. Science 259, 1742–1745.
[8] Kontsek, P. and Kontsekova, E. (1997) Forty years of interferon. Acta Virol. 41, 349–353.
[9] Biron, C.A. (1997) Natural killer cell regulation during viral infection. Biochem. Soc. Trans. 25, 687–690.
[10] Karupiah, G., Xie, Q.W., Buller, R.M., Nathan, C., Duarte, C. and MacMicking, J.D. (1993) Inhibition of viral replication by interferon-gamma-induced nitric oxide synthase. Science 261, 1445–1448.
[11] Lin, Y.L., Huang, Y.L., Ma, S.H., Yeh, C.T., Chiou, S.Y., Chen, L.K. and Liao, C.L. (1997) Inhibition of Japanese encephalitis virus infection by nitric oxide: antiviral effect of nitric oxide on RNA virus replication. J. Virol. 71, 5227–5235.
[12] Harris, N., Buller, R.M. and Karupiah, G. (1995) Gamma interferon-induced, nitric oxide-mediated inhibition of vaccinia virus replication. J. Virol. 69, 910–915.
[13] Katze, M.G., He, Y. and Gale, M. (2002) Viruses and interferon: A fight for supremacy. Nat. Rev. Immunol. 2, 675–687.
[14] Garcia-Sastre, A. (2002) Mechanisms of inhibition of the host interferon alpha/beta-mediated antiviral responses by viruses. Microbes Infect. 4, 647–655.
[15] Navarre, W.W. and Schneewind, O. (1999) Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. Microbiol. Mol. Biol. Rev. 63, 174–229.
[16] Mattsson, E., Verhage, L., Rollof, J., Fleer, A., Verhoef, J. and van Dijk, H. (1993) Peptidoglycan and teichoic acid from *Staphylococcus epidermidis* stimulate human monocytes to release tumour necrosis factor-alpha, interleukin-1 beta and interleukin-6. FEMS Immunol. Med. Microbiol. 7, 281–287.
[17] Masihi, K.N. (2000) Immunomodulators in infectious diseases: panoply of possibilites. Int. J. Immunopharmacol. 22, 1083–1091.
[18] Guidotti, L.G. and Chisari, F.V. (2001) Noncytolytic control of viral infections by the innate and adaptive immune response. Annu. Rev. Immunol. 19, 65–91.
[19] Ikeda, S., Negishi, T. and Nishimura, C. (1985) Enhancement of non-specific resistance to viral infection by muramyldipeptide and its analogs. Antiviral Res. 5, 207–215.
[20] Earl, P.L., Cooper, N., Wyatt, L.S., Moss, B. and Carroll, M.W. (1998) Preparation of cell cultures and vaccinia virus stocks. In: Current Protocols in Molecular Biology (Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K., Eds.), vol. 3, pp. 16.16.1–16.16.5. John Wiley & Sons, Inc., USA.
[21] Earl, P.L., Cooper, N., Wyatt, L.S. and Moss, B. (1998) Titration of vaccinia virus stocks by plaque assay. In: Current Protocols in Molecular Biology (Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K., Eds.), vol. 3, pp. 16.16.5–16.16.6. John Wiley & Sons, Inc., USA.
[22] Bach, E.A., Szabo, S.J., Dighe, A.S., Ashkenazi, A., Aguet, M., Murphy, K.M. and Schreiber, R.D. (1995) Ligand-induced autoregulation of IFN-gamma receptor beta chain expression in T helper cell subsets. Science 270, 1215–1218.
[23] Broide, D., Schwarze, J., Tighe, H., Gifford, T., Nguyen, M.D., Malek, S., Van Uden, J., Martin-Orozco, E., Gelfand, E.W. and Raz, E. (1998) Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. J. Immunol. 161, 7054–7062.
[24] Signoretto, C., del Mar, L.M., Tafi, M.C. and Canepari, P. (2000) Cell wall chemical composition of *Enterococcus faecalis* in the viable but nonculturable state. Appl. Environ. Microbiol. 66, 1953–1959.
[25] Popov, S.G., Villasmil, R., Bernardi, J., Grene, E., Cardwell, J., Popova, T., Wu, A., Alibek, D., Bailey, C. and Alibek, K. (2002) Effect of *Bacillus anthracis* lethal toxin on human peripheral blood mononuclear cells. FEBS Lett. 527, 211–215.
[26] Chen Jr., P.S., Toribara, T.Y. and Warner, H. (1956) Microdetermination of phosphorus. Anal. Chem. 28, 1756–1758.
[27] Solum, N.O. (1970) Some characteristics of the clottable protein of limulus polyphemus blood cells. Thromb. Diath. Haemorrh. 23, 170–181.
[28] Young, N.S., Levin, J. and Prendergast, R.A. (1972) An invertebrate coagulation system activated by endotoxin: evidence for enzymatic mediation. J. Clin. Invest. 51, 1790–1797.
[29] Ding, A.H., Nathan, C.F. and Stuehr, D.J. (1988) Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. J. Immunol. 141, 2407–2412.
[30] Liu, G., Zhai, Q., Schaffner, D., Bradburne, C., Wu, A., Hayford, A., Popov, S., Grene, E., Bailey, E. and Alibek, K. (2004) IL-15 induces IFN-beta and iNOS gene expression, and antiviral activity of murine macrophage RAW 264.7 cells. Immunol. Lett. 91 (2–3), 171–178.
[31] Keller, F. (1994) Methods for studying antiviral functions of macrophages and mononuclear phagocytes. J. Immunol. Meth. 174, 167–172.
[32] Di Marzio, P., Puddu, P., Conti, L., Belardelli, F. and Gessani, S. (1994) Interferon gamma upregulates its own gene expression in mouse peritoneal macrophages. J. Exp. Med. 179, 1731–1736.
[33] Fultz, M.J., Barber, S.A., Dieffenbach, C.W. and Vogel, S.N. (1993) Induction of IFN-gamma in macrophages by lipopolysaccharide. Int. Immunol. 5, 1383–1392.
[34] Martinez, D., Lynch, R.J., Meeker, J.B. and Field, A.K. (1980) Macrophage dependence of polyriboinosinic acid-polyribocytidylic acid-induced resistance to herpes simplex virus infection in mice. Infect. Immun. 28, 147–153.
[35] Morahan, P.S. and Morse, S.S. (1979) Macrophage virus interactions. In: Virus–lymphocyte Interactions: Implications for Disease (Aruffitt, M.R., Ed.), vol. 17. Elsevier/North Holland, New York.
[36] LeBlanc, P.A. (1989) Macrophage activation for cytolysis of virally infected target cells. J. Leukoc. Biol. 45, 345–352.
[37] Stohlman, S.A., Brayton, P.R., Fleming, J.O., Weiner, L.P. and Lai, M.M. (1982) Murine coronaviruses: isolation and characterization of two plaque morphology variants of the JHM neurotropic strain. J. Gen. Virol. 63, 265–275.
[38] Koff, W.C., Showalter, S.D., Seniff, D.A. and Hampar, B. (1983) Lysis of herpesvirus-infected cells by macrophages activated with free or liposome-encapsulated lymphokine produced by a murine T cell hybridoma. Infect. Immun. 42, 1067–1072.
[39] Stanwick, T.L., Campbell, D.E. and Nahmias, A.J. (1982) Cytotoxic properties of human monocyte-macrophages for human fibroblasts infected in herpes simplex virus: interferon production and augmentation. Cell. Immunol. 70, 132–147.
[40] Barot-Ciorbaru, R., Linna, T.J., Patel, M.R., Altman, J. and Carnaud, C. (1989) Enhancement of natural killer cell activity by *Nocardia opaca* fractions. Scand. J. Immunol. 29, 133–141.
[41] Pozur, V.K., Rashevskaia, E.V., Lazarenko, L.N., Krivokhatskaia, L.D. and Spivak, N.I. (1994) The effect of staphylococcal

peptidoglycan on macrophage interferon formation and bactericidal activity. Zh. Mikrobiol. Epidemiol. Immunobiol., 83–86.
[42] Pugin, J., Heumann, I.D., Tomasz, A., Kravchenko, V.V., Akamatsu, Y., Nishijima, M., Glauser, M.P., Tobias, P.S. and Ulevitch, R.J. (1994) CD14 is a pattern recognition receptor. Immunity 1, 509–516.
[43] Yoshimura, A., Lien, E., Ingalls, R.R., Tuomanen, E., Dziarski, R. and Golenbock, D. (1999) Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. J. Immunol. 163, 1–5.
[44] Fenton, M.J., Vermeulen, M.W., Kim, S., Burdick, M., Strieter, R.M. and Kornfeld, H. (1997) Induction of gamma interferon production in human alveolar macrophages by *Mycobacterium tuberculosis*. Infect. Immun. 65, 5149–5156.
[45] Pabst, M.J., Beranova-Giorgianni, S. and Krueger, J.M. (1999) Effects of muramyl peptides on macrophages, monokines, and sleep. Neuroimmunomodulation 6, 261–283.
[46] Masihi, K.N. (2000) Immunomodulatory agents for prophylaxis and therapy of infections. Int. J. Antimicrob. Agents 14, 181–191.
[47] Masihi, K.N., Kroger, H., Lange, W. and Chedid, L. (1989) Muramyl peptides confer hepatoprotection against murine viral hepatitis. Int. J. Immunopharmacol. 11, 879–886.
[48] Masihi, K.N. (1991) Highlights of the international symposium on immunotherapeutic prospects of infectious diseases. Nat. Immun. Cell Growth Regul. 10, 185–195.
[49] Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K. and Akira, S. (1999) Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 11, 443–451.
[50] Goguel, A.F., Payelle, B., Quan, P.C. and Lespinats, G. (1984) Stimulation of one particular subset of natural killer cells by peptidoglycans extracted from gram-positive bacteria. J. Natl. Cancer Inst. 73, 697–703.